The zinc finger transcription factor Early Growth Response 2 (Egr-2) is an intrinsic regulator of T cell tolerance and homeostasis

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Declaration

I, Alistair Symonds, confirm that I am the author of this work and that where information has been obtained from other sources I have acknowledged this within the text or below.

The work presented here on the Egr-2 cKO and Tg mice was performed jointly by the student and Dr B. Zhu. The cloning required to establish the Egr-2 Tg was performed by Dr B. Zhu while microinjection was performed by Mr W. Mansfield of the Transgenic core facility, Queen Mary, University of London. For all experiments lymphoid organ extraction was performed by Dr B. Zhu. In addition, genotyping of Egr-2 cKO (Figure 3.2, A) and Tg mice (Figure 3.3, A), the analyses of Egr-2 cKO $CD4^+$ T cell proliferation *in vitro* (Figure 3.10, A) and *in vivo* (Figure 3.13) and the analyses of ERK phosphorylation (Figure 3.10, B) and FasL expression (Figure 3.11, C) were performed by Dr B. Zhu. For other experiments involving Egr-2 cKO and Tg mice the student and Dr B. Zhu performed independent experiments.

In addition, the histological examinations of the diseased mice were performed by Professor J. Martin and colleagues at the Pathology department, Queen Mary, University of London (Figure 3.16, B and D). Furthermore, the microarray sample labelling and array hybridisation was performed by the Genome Centre, Queen Mary, University of London. Results analysis was performed by the student. FACS based separation of $CD4^+CD25^-CD44^{low}$ and $CD4^+CD25^-CD44^{high}$ cells was performed by Dr G. Warnes of the Flow Cytometry core facility, Queen Mary, University of London. Samples from Tg4 Tg mice were obtained from Professor D. Wraith and colleagues at Bristol University.

Finally, the experiments involving adoptive transfer of T cells [(Figure 3.14) and (Figure 3.17, C)] were performed by Dr S. Li and colleagues at Brunel University. In addition, the analysis of antibodies in the serum of Egr-2 cKO mice (Figure 3.17, A and B), the analyses of proteinuria and deposition of immune complexes in the glomeruli (Figure 3.16, C and E), the analyses of intracellular cytokine production by $CD4^+$ T cells from mice of different ages (Figure 3.28, A) and by $CD44^{low}$ and $CD44^{high}$ $CD4^+$ T cells (Figure 3.28, B), the PCR analyses of Egr-2 expression in B cells from Egr-2 cKO mice (Figure 3.2, D) and in thymocytes from Egr-2 Tg mice (Figure 3.3, B) were also performed at Brunel by Dr S. Li and colleagues.

Signature of student:.....

Signature of supervisor:.....

Abstract

Tolerance of T cells to self-antigen is crucial to prevent the development of autoimmune disease. How self-tolerance is controlled at the transcriptional level is, however, unknown. We discovered that the transcription factor Early Growth Response 2 (Egr-2) was expressed by tolerant T cells, and by $CD4^{+}CD44^{high}$ T cells in the absence of overt antigen stimulation, *in vivo*. To investigate the roles of Egr-2 in T cells, we generated CD2 cell specific Egr-2 deficient (Egr-2 cKO) mice. The proliferation of Egr-2 cKO $CD44^{high}$ T cells *in vivo* was markedly increased leading to progressive accumulation as the mice aged. By 15 months of age $CD4^{+}CD44^{high}$ cells constituted the predominant T cell population in the peripheral lymphoid organs of Egr-2 cKO mice and expressed high levels of the activation markers CD25 and CD69. In addition to this lymphoproliferative disorder, 15 month old Egr-2 cKO mice showed signs of lupus-like autoimmune disease. This autoimmune syndrome was characterised by glomerulonephritis and proteinuria, infiltration of T cells into internal organs and, crucially, auto-antibodies directed against nuclear components; the hallmark of lupus. We observed decreased expression of the cyclin-dependent kinase inhibitor p21cip1 in Egr-2 cKO $CD4^{+}CD44^{high}$ T cells while TCR stimulation induced IFN-γ, and, in particular, IL-17A and IL-17F expression was markedly increased. Consistent with these findings, we observed increased numbers of IFN-γ and IL-17 producing $CD4^{+}$ T cells in Egr-2 cKO mice. The numbers of IFN-γ and IL-17 producing $CD4^{+}$ T cells further increased as the mice aged in parallel with the gradual development of symptoms of lupus-like disease. These results demonstrate that Egr-2 is an intrinsic regulator of both T cell homeostasis and T cell tolerance.

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Abbreviations

AICD	Activation induced cell death
AP-1	Activator protein 1
APC	Antigen presenting cell
Bcl-2	B cell lymphoma 2
Bcl-X_L	B cell lymphoma X, long isoform
CD	Cluster of differentiation
DKO	Double knockout
DN	Double negative
DP	Double positive
EGFP	Enhanced green fluorescent protein
Egr	Early growth response
ERK	Extracellular signal regulated kinase
FoxP3	Forkhead box P3
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasal
ISP	Immature single positive
KO	Knockout
LCR	Locus control region
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor of kappa-light-chain-enhancer of activated B cells
NK	Natural killer
PD-1	Programmed death 1
RAG	Recombination activating gene
SOCS	Suppressor of cytokine signalling
SP	Single positive
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T cell receptor
Tg	Transgenic
TNF	Tumour necrosis factor

Chapter 1: INTRODUCTION

The immune system provides protection from infection by pathogenic microorganisms and viruses. The importance of this system is demonstrated by the severity of acquired and congenital immunodeficiencies. The immune system is divided into two arms; the innate and adaptive systems. While the innate system responds in an identical fashion each time it encounters a given pathogen, the adaptive immune system responds more rapidly upon re-exposure to the same pathogen. A vital part of the adaptive system is cells called lymphocytes. The two main subsets of lymphocytes are B cells and T cells. B cells produce immunoglobulins (Ig), or antibodies, that are vitally important in host defence, while the various subsets of T cells serve a myriad of roles. Classical T cells are defined by the expression of a T Cell Receptor (TCR) consisting of α and β chains (Allison *et al.*, 1982; Haskins *et al.*, 1983; Kappler *et al.*, 1983; Hedrick *et al.*, 1984) and these αβ T cells are generally confined to the lymphatic system in the absence of pathogen infection. Conversely, T cells bearing a TCR composed of γ and δ chains are non-classical and these γδ T cells are located in peripheral tissues even in the absence of an active infection (Koning *et al.*, 1987; Stingl *et al.*, 1987; Goodman and Lefrancois, 1988). The two main subsets of classical αβ T cells are distinguished by the expression of the markers CD4 and CD8 (Cantor and Boyse, 1975). $CD8^+$ T cells can kill cells that have been infected with viruses while $CD4^+$ T cells orchestrate the immune response as they can provide assistance to other cells types such as B cells and macrophages (Cantor and Boyse, 1975).

Unlike B cells that recognise intact antigen in its native conformation, αβ T cells recognise antigen as processed peptides in the context of Major Histocompatibility

Complex (MHC) molecules (Watts *et al.*, 1984; Babbitt *et al.*, 1985). $CD4^+$ T cells and $CD8^+$ T cells are restricted to recognising antigen as peptides loaded on MHC class II and MHC class I respectively (Okada and Henney, 1980; Meuer *et al.*, 1982). Unlike class I molecules that are ubiquitously expressed, class II molecules are confined to a small number of "professional" antigen presenting cells (APCs) such as dendritic cells which take up and process exogenous antigen for presentation on class II.

MHC molecules can bind both self and non-self peptides. T cells need to discriminate between self and non-self so as to provide protective immunity against the latter but not react against the former. This state of non-reactivity to self is termed self-tolerance and is vital to avoid autoimmune destruction of self-tissues. As many other cells require help from $CD4^+$ T cells, tolerance induction in $CD4^+$ T cells is very important. Most of the self-reactive $CD4^+$ T cells are eliminated during their development in the thymus in a process termed central tolerance (Kappler *et al.*, 1987; Ashton-Rickardt *et al.*, 1994). However, a few self-reactive $CD4^+$ T cells do escape from central tolerance and are controlled in the periphery via peripheral tolerance mechanisms (Liu *et al.*, 1995).

1.1 T cell development and central tolerance

T cells are derived from progenitors in the bone marrow which migrate to the thymus where they develop into mature T cells. A unique feature of T, and also B, lymphocytes is that the molecules they use for pathogen detection are not germline encoded; instead they are generated by recombination of DNA segments (Early *et al.*, 1980). During T cell development in the thymus the T cell precursors, called thymocytes, rearrange their TCR genes. A mature αβ TCR consists of both α and β chains; the genes encoding both of these chains are rearranged during T cell development. The Variable (V) region of

both the TCR α and β genes consists of multiple gene segments which are spliced together to form the final complete V region exon. Both α and β genes have Variable (V) and Joining (J) segments while the β gene also has Diversity (D) segments. Proteins called Recombination Activating Genes (RAG) 1 and 2 direct the recombination machinery to the TCR loci resulting in the splicing of these V(D)J segments together to form the mature V region exon (Schatz *et al.*, 1989; Oettinger *et al.*, 1990; Shinkai *et al.*, 1992). Each recombination event involves the breakage and rejoining of the DNA sequence. Each time the DNA is broken and rejoined additional nucleotides can be incorporated into the DNA sequence. The addition of nucleotides is random and, if the number of inserted nucleotides is not a multiple of 3, can result in a frameshift mutation and a non-functional TCR α or β chain polypeptide. Thus, selection events are required to select only those cells that have functional TCR chains. In addition, thymocytes are selected for cells that weakly recognise self-MHC molecules loaded with self-peptide (Ashton-Rickardt *et al.*, 1994; Hogquist *et al.*, 1994). These steps that select only those cells that have a functional β chain and those cells that recognise self-peptide/MHC molecules are termed β-selection and positive selection respectively. Furthermore, due to the nature of the process that generates TCR chains, potentially auto-reactive cells are generated. During T cell development many of these potentially autoreactive cells are deleted in a process called negative selection (Kappler *et al.*, 1987; Ashton-Rickardt *et al.*, 1994). This series of selection events is termed thymic education.

1.1.1 Double Negative cells and β-selection

Initially, thymocytes express neither CD4 nor CD8 and are referred to as Double Negative (DN) cells (see Figure 1.1). The DN cell stage can be subdivided into 4 stages based on the expression of the cell surface markers CD25 and CD44 (Godfrey *et al.*,

1993). The earliest DN stage, termed DN1, expresses only CD44, while the next stage, DN2, expresses both CD25 and CD44 (Godfrey *et al.*, 1993). DN3 cells express CD25 but not CD44, while the final DN stage, DN4, expresses neither CD25 nor CD44 (Godfrey *et al.*, 1993). During the DN stages the thymocyte rearranges its TCR β genes. Due to the possibility of producing a non-functional β chain, the developing T cells undergo a process of selection which permits only those cells that possess a successfully rearranged β gene to develop further. This step is referred to as the β-selection checkpoint and occurs at the DN3 to DN4 transition. The β chain is transcribed from the rearranged β locus and pairs with an invariant germline encoded pre-TCRα chain (Groettrup *et al.*, 1993; Fehling *et al.*, 1995; Fehling *et al.*, 1997). If the rearranged β chain polypeptide can successfully form a heterodimer with the pre-TCRα chain the complex signals through associated CD3 chains and the cell is selected to further develop (Groettrup *et al.*, 1993; Fehling *et al.*, 1995; Fehling *et al.*, 1997).

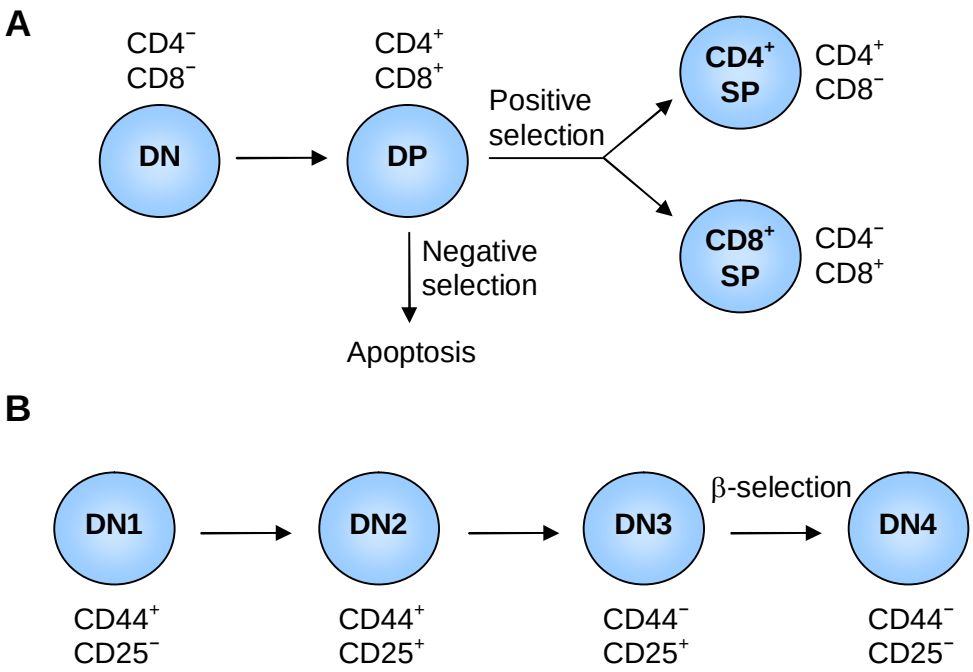

Figure 1.1: T cell development. During T cell development in the thymus the T cell precursors, called thymocytes, pass through a series of stages which can be identified by their cell surface markers. The major thymocyte subsets are distinguished by their differential expression of CD4 and CD8 (**A**). Initially, the thymocytes express neither CD4 nor CD8 and are termed Double Negative cells (DN). The $CD4^-CD8^-$ DN stage can be further subdivided based on the expression of CD44 and CD25 (**B**). See text for further details. Based on figures from (Janeway *et al.*, 2001).

1.1.2 Double Positive cells and Positive and Negative selection

The thymocytes that have successfully rearranged their TCR β genes proceed from the DN stage to an intermediate stage called Immature Single Positive (ISP) (Miyazaki, 1997). These cells express CD8 but are immature and do not express a TCR complex. In normal mice the cells quickly pass through the ISP stage to the next major stage of T cell development: the Double Positive (DP) stage. DP cells express both CD4 and CD8 and it is at this stage that the cells rearrange their TCR α genes and undergo a number of further selection events (see Figure 1.1). These selection events are dependent on the

ability of the thymocyte's TCR to recognise self-MHC molecules loaded with self-peptide. Thymocytes that do not recognise self-peptide/MHC complexes at all receive no stimulation and die from "neglect". Thymocytes possessing a TCR that strongly recognises self-peptide/MHC complexes are deleted by apoptosis; this process is termed negative selection (Kappler *et al.*, 1987; Ashton-Rickardt *et al.*, 1994). Only thymocytes that weakly recognise self-peptide/MHC complexes survive and mature into naïve $CD4^+$ or $CD8^+$ T cells (Ashton-Rickardt *et al.*, 1994; Hogquist *et al.*, 1994). This step is referred to as positive selection. Hence there are two thresholds; if the TCR stimulus exceeds the first the thymocyte is selected to become a mature cell but if the second is also exceeded the cell is deleted (Ashton-Rickardt *et al.*, 1994). This removal of highly self-reactive T cells from the repertoire by negative selection is termed central tolerance.

1.2 Mature T cells and peripheral tolerance

Once T cells have completed their maturation in the thymus they migrate to the periphery. Although these T cells are mature, they are termed immunologically naïve as they have not been activated in the periphery. These naïve T cells express molecules, such as CD62L (Gallatin *et al.*, 1983), that facilitate their circulation through the blood and secondary lymphoid tissues such as lymph nodes and the white pulp of the spleen. In these secondary lymphoid tissues the naïve T cells encounter APC presenting peptides on MHC molecules. The interaction with APC can induce one of several different responses in the T cells. The level of stimulation that the T cell receives upon interaction with the APC presenting peptide-MHC molecules determines the response of that T cell. If the T cell receives a strong signal, such as might be delivered upon interaction with an APC presenting a foreign peptide, then the T cell will become

activated and induce an immune response. If the T cell receives only a weak signal, such as typically occurs when encountering an APC presenting self-antigen, then it will not induce an immune response but such interactions may be important for the cell's survival and homeostasis. However, while central tolerance induces the deletion of highly auto-reactive T cells (see section 1.1), some potentially auto-reactive cells with a relatively low avidity for self-antigen escape central tolerance (Liu *et al.*, 1995). These potentially auto-reactive T cells are controlled in the periphery in healthy individuals by various peripheral tolerance mechanisms.

1.2.1 T cell activation

If the antigen presented by the APC is of sufficiently high affinity it will induce a strong signal in the T cell which is sufficient to surpass the threshold for T cell activation. In such cases a cascade of signalling molecules is activated which, in turn, leads to the generation or release of second messengers, such as Ca^{2+}. This signalling cascade eventually results in the induction of transcription factors, such as members of the Fos/Jun (Halazonetis *et al.*, 1988; Sassone-Corsi *et al.*, 1988), Nuclear Factor of Activated T cells (NFAT) (Shaw *et al.*, 1988) and Early Growth Response (Egr) (Harris *et al.*, 2004; Safford *et al.*, 2005; Anderson *et al.*, 2006) protein families (see Figure 1.2). These transcription factors initiate the process of proliferation and also induce the expression of the cytokine Interleukin-2 (IL-2). IL-2 is a T cell growth factor that can act in an autocrine fashion to promote the proliferation of the cell that produces it (Ruscetti *et al.*, 1977). This forms the basis of two of the main assays of $CD4^+$ T cell activation; analysis of proliferation and IL-2 secretion.

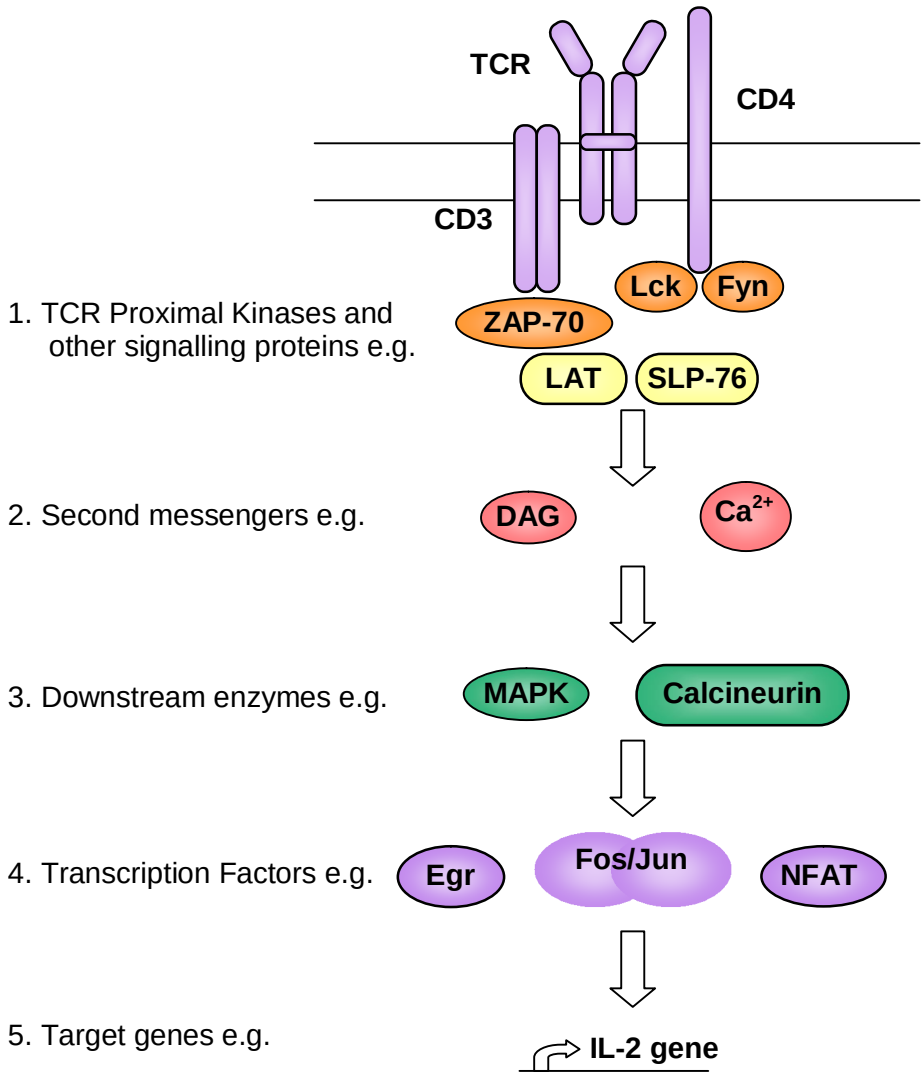

Figure 1.2: Schematic of TCR signalling. Upon engagement of the TCR with a high affinity peptide-MHC ligand a signalling cascade is activated that results in T cell activation. The Src family kinases Lck and Fyn phosphorylate Immunoreceptor Tyrosine Activation Motifs (ITAMs) in the CD3 and ζ chains that are associated with the TCR. This creates docking sites for other signalling proteins the action of which leads to the generation or release of second messengers such as DiAcylGlycerol (DAG) and Ca^{2+}. These in turn lead to the activation of downstream enzymes such as the MAPKs (Mitogen Activated Protein Kinases) and the Ca^{2+} dependent phosphatase Calcineurin. These downstream effectors induce the activation of a cascade of transcription factors including members of the NFAT (Nuclear Factor of Activated T cells), Fos/Jun and Egr protein families. These transcription factors induce the expression of genes involved in T cell activation and proliferation such as IL-2. N.B This schematic is greatly simplified and many crucial signalling molecules are omitted. Based on figures from (Janeway *et al.*, 2001).

Following initial antigen encounter $CD4^+$ T cells differentiate into effector cells which provide cytokines and other signals to other cell types such as B cells and macrophages. Based upon the cytokines produced these effector cells can be classified into different categories. $CD4^+$ T cells that secrete the cytokine Interferon gamma (IFN-γ) are termed T_H1 cells while those that produce the cytokine Interleukin 4 (IL-4) are designated T_H2 cells (see Murphy and Reiner, 2002). $CD4^+$ T cells that secrete IL-17 (also called IL-17A) have recently been reported to represent a distinct type of effector cell and are often referred to as T_H17 cells (see Dong, 2008). Furthermore, $CD4^+$ T cells that provide help to B cells in germinal centres, which have been designated T Follicular Helper (T_{FH}) cells, are another type of effector cell although their relationship to other T cell subsets remains unclear (see Vinuesa *et al.*, 2005). These T_{FH} cells are not defined by their cytokine profile but by their expression of the chemokine receptor CXCR5 which promotes localisation to lymphoid follicles in secondary lymphoid tissues (see Vinuesa *et al.*, 2005). However, while distinct T_H1, T_H2 and T_H17 cells can be generated using specific culture conditions *in vitro*, the situation is not as clear-cut *in vivo* and there appears to be a degree of plasticity. $CD4^+$ T cells that produce both IFN-γ and IL-17 have been observed in many models (see Dong, 2008) while IL-17 production by cells with a T_{FH} phenotype has also been documented (Hsu *et al.*, 2008; Wu *et al.*, 2008).

These various subsets of effector cells provide assistance to other cell types such as B cells and macrophages and thereby help to resolve the infection. After the inciting antigen has been eliminated, the majority of the responding T cells die but a small cohort of cells persists. These cells, termed memory cells, can respond more rapidly than naïve T cells upon re-exposure to the same antigen *in vivo* (Veiga-Fernandes *et al.*, 2000).

1.2.2 T cell homeostasis

The total number of T cells present in the periphery remains relatively constant over time despite the influx of newly matured T cells, the numbers of which varies considerably with age, from the thymus (see Surh and Sprent, 2008). This implies the existence of mechanisms that serve to maintain T cell numbers and to support the survival of individual cells. This maintenance of a constant total number of T cells is termed T cell homeostasis. Hence phenomena such as the deletion of the majority of the responding T cells at the termination of an immune response and the hyper-proliferation of T cells in response to lymphopaenia can both be classified as homeostatic mechanisms but I will focus here on the maintenance of T cell populations in the steady state.

Initial studies of T cell homeostasis focussed on the role of peptide-MHC in the maintenance of T cells (Kirberg *et al.*, 1997; Ernst *et al.*, 1999). Several early studies concluded that the self-peptide/MHC ligands responsible for positive selection in the thymus were also necessary for the survival of mature but immunologically naïve T cells in the periphery (Kirberg *et al.*, 1997; Ernst *et al.*, 1999). However, these studies used adoptive transfer of T cells into lymphopaenic recipients to demonstrate this and were therefore complicated by the concomitant proliferation that occurs in lymphopaenia (see Surh and Sprent, 2008). Subsequently, two reports found that transferred naïve $CD4^+$ T cells survived for at least 4-6 weeks in I-A$\beta^{-/-}$ mice on a H-2^b background (Clarke and Rudensky, 2000; Dorfman *et al.*, 2000). Since mice on this background have a point mutation in the I-Eα gene, H-2^b I-A$\beta^{-/-}$ mice have traditionally been considered MHC class II deficient and hence the authors concluded that interactions with MHC class II molecules are not required for the survival of naïve

$CD4^+$ T cells in the periphery (Clarke and Rudensky, 2000; Dorfman *et al.*, 2000). However, this interpretation has been called into question by recent findings: two independent groups found considerable differences in the responses of T cells in H-2^b I-A$\beta^{-/-}$ mice and mice that have null mutations in all 4 MHC class II chain genes (Martin *et al.*, 2003; Purton *et al.*, 2007) which has been attributed to the formation of an heterodimer from the remaining I-Aα and I-Eβ chains in H-2^b I-A$\beta^{-/-}$ mice (Martin *et al.*, 2003). Therefore, interactions with this non-classical MHC molecule may have supported the homeostasis of T cells in H-2^b I-A$\beta^{-/-}$ mice and the survival of the T cells in these mice does not exclude a role for peptide-MHC in naïve $CD4^+$ T cell homeostasis. More recent work has demonstrated that the half-life of naïve $CD4^+$ T cells in nonlymphopaenic mice is increased two fold in the presence of MHC class II molecules (Martin *et al.*, 2006). Therefore, collectively the data suggests that interactions with peptide-MHC complexes are involved in the survival of both naïve $CD4^+$ and naïve $CD8^+$ T cells in normal conditions. Consistent with this, peripheral ablation of the expression of both of the Src family kinases Lck and Fyn, which are required for signal transduction downstream of the TCR, resulted in the gradual disappearance of naïve T cells (Seddon and Zamoyska, 2002). Thus, TCR signals, presumably induced by interactions with peptide-MHC, are necessary for the survival of naïve T cells. However, TCR signals are not the only factor involved in the homeostasis of naïve T cells. Several studies have found that the IL-2 related cytokines IL-7 and IL-15 are also involved in the homeostasis of naïve T cells in the periphery (Kennedy *et al.*, 2000; Schluns *et al.*, 2000; Tan *et al.*, 2001; Berard *et al.*, 2003). While IL-7 is important for both naïve $CD4^+$ and naïve $CD8^+$ T cells, IL-15 is apparently only involved in the survival of naïve $CD8^+$ T cells (Kennedy *et al.*, 2000; Berard *et al.*, 2003). Therefore, for naïve T cells TCR signals coupled with the cytokine IL-7, and also IL-15 for naïve $CD8^+$ T cells, jointly regulates their survival.

However, it has become apparent that those cells that have encountered antigen are controlled differently. The marker CD44 is expressed at low levels on immunologically naïve cells but is upregulated after antigen encounter. Therefore $CD44^{high}$ cells are generally considered to be effector cells or memory cells (see Surh and Sprent, 2008). Two different approaches have been used to investigate the homeostatic requirements of $CD44^{high}$ T cells and these have yielded different results. The first approach uses a monoclonal T cell population, i.e. expressing a specific TCR, generated by transgenic techniques (see section 1.2.3.1.1) coupled with intentional immunisation and are termed "antigen specific memory" cells (see Surh and Sprent, 2008). The alternative approach uses a polyclonal population of $CD44^{high}$ T cells, where the T cells express a range of different TCRs, isolated from mice that have not been intentionally immunised. These $CD44^{high}$ cells are presumably generated in response to self-antigen or environmental antigens, such as commensal flora, and have been designated "memory phenotype" or "MP" cells (see Surh and Sprent, 2008). Studies using antigen specific memory cells found that these cells still persist in the absence of MHC molecules (Murali-Krishna *et al.*, 1999; Swain *et al.*, 1999). Therefore, it was concluded that peptide-MHC ligands are not required for the survival of $CD44^{high}$ cells and that instead $CD44^{high}$ cells require the cytokines IL-7 and IL-15 (Kennedy *et al.*, 2000; Schluns *et al.*, 2000; Berard *et al.*, 2003). While most studies of MP $CD8^{+}$ $CD44^{high}$ cells concur with this view, one report found that a subset of the MP $CD8^{+}$ $CD44^{high}$ population was dependent on the presence of MHC class I for their homeostasis (Boyman *et al.*, 2006). In addition, the homeostatic proliferation of MP $CD4^{+}CD44^{high}$ cells is reduced in the absence of TCR signalling (Seddon *et al.*, 2003). Whether this reflects a role for peptide-MHC for the homeostasis of all $CD4^{+}CD44^{high}$ T cells (Seddon *et al.*, 2003; Robertson *et al.*, 2006) or only for particular subsets of this population (Purton *et al.*, 2007) remains unclear. In

any case, these results demonstrate that, in addition to the cytokines IL-7 and IL-15, TCR signals are involved in the homeostasis of at least some subsets of $CD44^{high}$ cells.

1.2.3 Peripheral T cell tolerance

Potentially self-reactive T cells do escape central tolerance (see section 1.1) due to a relatively low avidity for self-peptide/MHC (Liu *et al.*, 1995) and migrate to the periphery where they could potentially cause autoimmune disease. In healthy individuals these T cells are effectively controlled by various mechanisms that are collectively termed peripheral tolerance. These mechanisms fall into two categories; cell extrinsic, where the activation or effector function of the cell is suppressed by other cells, and cell intrinsic, where the cell either does not respond or dies due to inherent mechanisms. An important cell extrinsic mechanism is the suppression of T cell responses by regulatory T (T_{Reg}) cell populations such as $CD4^+CD25^+FoxP3^+$ T_{Reg} cells and T_R1 cells. $CD4^+CD25^+FoxP3^+$ T_{Reg} cells are a subset of $CD4^+$ T cells that can inhibit the proliferation of other T cells and the development of autoimmune disease (Fontenot *et al.*, 2003; Hori *et al.*, 2003). While the suppressive activity of $CD4^+CD25^+FoxP3^+$ T_{Reg} cells is now well established, the mechanisms that these cells utilise to inhibit other cells are less clear. T_R1 cells respond to TCR stimulation by secreting the anti-inflammatory cytokine IL-10 and are capable of inhibiting the proliferation of other T cells and the induction of autoimmune disease (Groux *et al.*, 1997). T_R1 cells were first induced *in vitro* by chronic antigen stimulation in the presence of IL-10 (Groux *et al.*, 1997) but similar cells can be induced *in vivo* by repeated antigen administration (Sundstedt *et al.*, 2003). Immune deviation is another process that can regulate immune responses but this influences effector function rather than activation. Immune deviation is the process where one T cell subset regulates the

development and/or activity of another e.g. T_H1 and T_H2 cells secrete cytokines, IFN-γ and IL-4 respectively, that negatively regulate the development of the other subset. The other type of peripheral tolerance mechanism is cell intrinsic and includes ignorance, deletion/Activation Induced Cell Death (AICD) and anergy. The cell intrinsic mechanisms are, like thymic education, dependent on the level of stimulation that the cell receives upon encountering antigen. This includes not only TCR signalling ("signal 1") but also the "second signals" provided by accessory molecules present on the surface of APCs. These second signals can be either stimulatory, such as ligation of CD28 by B7 molecules, or inhibitory such as those transduced by PD-1 upon interaction with PD-L1 or PD-L2 (see Sharpe and Freeman, 2002). These two types of second signal are termed co-stimulation and co-inhibition respectively. In addition the cytokines present in the environment can also influence T cell activation. If the level of the self-peptide/MHC is low the TCR signal will not surpass the threshold for activation and will be insufficient to induce effector functions; the T cell is said to be ignorant of the antigen. However, as mentioned above (see section 1.2.2) such covert interactions with self-peptide/MHC have an important role in maintaining the peripheral T cell repertoire (Kirberg *et al.*, 1997; Ernst *et al.*, 1999; Seddon and Zamoyska, 2002; Surh and Sprent, 2008). AICD occurs when the T cell is activated through the TCR and proliferates but receives such a strong signal that the progeny die by apoptosis. This is thus analogous to negative selection in the thymus.

1.2.3.1 Anergy and adaptive tolerance

Anergy is a state of T cell hyporesponsiveness to antigen where proliferation and IL-2 production in response to TCR stimulation is impaired, but the cells remain alive (see Schwartz, 2003). Various types of anergy have been documented and have been

classified by Schwartz into 2 broad categories: clonal anergy and adaptive tolerance; the former has only been documented *in vitro* while the latter is an *in vivo* state (2003) (see also Table 1.1). While in clonal anergy the cells merely seem to be unable to proliferate or produce IL-2, in adaptive tolerance effector functions also appear to be impaired (see Schwartz, 2003). The biochemical alterations in clonal anergy and adaptive tolerance are different indicating that the mechanisms responsible for their induction and maintenance are probably different (Anderson *et al.*, 2006; Chiodetti *et al.*, 2006). Clonal anergy can be induced *in vitro* by TCR ligation in the absence of co-stimulation (Jenkins *et al.*, 1990), while adaptive tolerance is induced *in vivo* in an environment that is presumably low in co-stimulation and/or high in co-inhibition (see Schwartz, 2003). In adaptive tolerance the T cells initially proliferate in response to antigen but after a few days many of these cells die with the remaining population down-regulating their responses. In addition in many models of adaptive tolerance the T cells respond to TCR stimulation by secreting the anti-inflammatory cytokine IL-10 in a similar fashion to T_R1 cells (see Schwartz, 2003). In the absence of antigen (e.g. transferring to a host not expressing the antigen), the responsiveness of the adaptively tolerant cells is restored indicating that the persistence of antigen is required to maintain the tolerant state (Tanchot *et al.*, 2001). However, transferring adaptively tolerant T cells into another host expressing higher levels of the same antigen leads to the induction of a more profound hyporesponsiveness (Tanchot *et al.*, 2001; Singh and Schwartz, 2003). It is these findings that led to the terminology of "adaptive" tolerance (see Schwartz, 2003) since the T cells appear to be changing their activation threshold in response to environmental signals.

	Clonal anergy	Adaptive tolerance
Induction	*in vitro*	*in vivo*
Induction signalling pathway	Ca^{2+}/NFAT	Unknown
Proliferation with induction	No	Yes
Impaired proliferation	Yes	Yes
Impaired IL-2 production	Yes	Yes
Impaired effector cytokine production	No	Yes
Production of IL-10	No	Some models
Major signalling pathways blocked	Ras/MAPK	Ca^{2+}/Tyr kinase
Antigen persistence required	No	Yes

Table 1.1: Comparison of clonal anergy and adaptive tolerance. Based on a more comprehensive table from (Schwartz, 2003).

Adaptive tolerance was first observed following injection of superantigens, which bind independently of the peptide binding regions of the MHC to a largely invariant part of the TCR (see Schwartz, 2003). However, due to the unusual nature of the TCR contact, whether this state could be induced with other antigens was unclear. Since then similar adaptive tolerance states have been induced in a number of different models with antigens that utilise a more conventional TCR contact. Schwartz and colleagues induced adaptive tolerance by transferring T cells expressing a transgenic TCR with affinity for pigeon cytochrome C into a T cell deficient host expressing the antigen (Tanchot *et al.*, 2001). Sarukhan and co-workers induced tolerance by crossing a TCR transgenic mouse containing T cells that respond to a peptide derived from haemaggulutinin (HA) with a mouse that expressed this antigen under the control of an Ig-κ promoter (Lanoue *et al.*, 1997). However, in this model the HA antigen would be expressed in the thymus so tolerance may have been at least partially established centrally. Adaptive tolerance has also been induced by repeated administration of peptide in a model developed by Wraith and colleagues.

1.2.3.1.1 Antigen-induced adaptive tolerance

This model was developed from studies on Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of Multiple Sclerosis. EAE is characterised by $CD4^+$ T cells directed against peptides from nervous system proteins such as myelin basic protein (MBP). Injection of spinal cord homogenate with a suitable adjuvant induces EAE in $H\text{-}2^u$ mice (Metzler and Wraith, 1993). However, one intranasal (i.n.) administration of the immunodominant peptide from MBP (Ac1-9), or a high affinity analogue (Ac1-9[4Y]), is sufficient to render the mice resistant to subsequent EAE induction (Metzler and Wraith, 1993). The low frequency of the T cells that respond to this peptide makes analysis of this population difficult; therefore a TCR transgenic mouse model was established (Liu *et al.*, 1995). The T cells that respond to Ac1-9 were isolated and their TCR α and β chain genes cloned. These cloned genes were inserted into constructs so that their expression was under the control of the human CD2 promoter and locus control region (LCR). These two constructs were then introduced into fertilised mouse ova which were then injected into pseudo-pregnant female mice. The resulting progeny in which these genes had integrated into the mouse genome were identified and served as founders for TCR transgenic lines. The hCD2 regulatory elements drive expression of the TCR α and β chain genes in developing thymocytes and in mature T cells of these TCR transgenic mice. One of these lines, termed Tg4, expresses the transgenic TCR β chain gene on more than 90% of developing $CD4^+$ SP thymocytes (Liu *et al.*, 1995). Thus, in these Tg4 TCR transgenic mice the majority of $CD4^+$ T cells bear a TCR with affinity for Ac1-9 hence providing a largely homogenous population of cells for analysis. These transgenic mice can be tolerised by repeated i.n. administration of Ac1-9[4Y] (Burkhart *et al.*, 1999). When these peptide induced tolerant $CD4^+$ T cells are stimulated with antigen *in vitro* they exhibit only minimal

proliferation and IL-2 production (Burkhart *et al.*, 1999); the hallmarks of adaptive tolerance. Instead the cells respond to TCR stimulation by secreting the anti-inflammatory cytokine IL-10 and can suppress the activation of other T cells which, at least *in vivo*, requires IL-10 (Sundstedt *et al.*, 2003). Thus these cells are not only hyporesponsive they also have suppressive capabilities.

1.2.3.1.2 Maintenance of adaptive tolerance

Antigen persistence is required to maintain the tolerant state suggesting that repeated TCR signalling is required to synthesise, or activate, so called "anergic factors" responsible for maintaining the hyporesponsive state of the cell (see Schwartz, 2003). Global analysis of gene expression in tolerant cells has revealed a distinct gene expression programme suggesting that this is regulated, at least in part, at the transcriptional level (Lechner *et al.*, 2001; Anderson *et al.*, 2006). However, the activation of many of the well characterised transcription factors that function in T cell activation, such as NF-κB and AP-1, is impaired in tolerant T cells and no transcription factors have so far been identified that are specific to the tolerant state (Anderson *et al.*, 2006; Chiodetti *et al.*, 2006). Therefore, the prevailing hypothesis is that the transcription factors that are involved in the establishment and maintenance of tolerance are also present during normal T cell activation but that they only serve to promote hyporesponsiveness in the absence of other signals (see Schwartz, 2003). Thus far, however, the identities of the transcription factors that are involved in the establishment and maintenance of the hyporesponsive state are unknown.

Recent studies of models of clonal anergy have identified the Nuclear Factor of Activated T cells (NFAT) family of transcription factors as important in the induction of clonal anergy.

1.2.3.1.3 NFAT and clonal anergy

In resting T cells NFAT proteins are heavily phosphorylated and localised to the cytosol and hence are unable to activate transcription. TCR engagement results in an increase in intracellular Ca^{2+} which, in turn, induces activation of the Ca^{2+}/calmodulin dependent phosphatase calcineurin. This phosphatase dephosphorylates the cytosolic phopshorylated form of NFAT. This dephosphorylation allows NFAT to translocate to the nucleus where it can initiate transcription of target genes. Ca^{2+} signalling is induced by TCR stimulation alone with little contribution from co-stimulatory pathways. Thus NFAT activation occurs under the conditions that promote clonal anergy induction making it a potential candidate for an anergic factor. Indeed, the drug cyclosporine and it's derivatives that function by inhibiting calcineurin and hence NFAT activation can inhibit clonal anergy induction (Jenkins *et al.*, 1990). Furthermore, Rao and colleagues found that clonal anergy was not induced in NFAT1 knockout (KO) T cells indicating that NFAT is essential for the induction of hyporesponsiveness (Macian *et al.*, 2002). Using a pharmacological model that mimics clonal anergy, Rao and colleagues proposed that the mechanism for NFAT function in the induction of hyporesponsiveness was upregulation of E3 Ubiquitin ligase expression (Heissmeyer *et al.*, 2004). E3 Ubiquitin ligases are enzymes that target specific proteins and tag them with the small peptide Ubiquitin. Such Ubiquitylated proteins are then recognised by the proteasesome which initiates their degradation. Thus E3 Ubiquitin ligases target specific proteins for degradation. Among the E3 Ubiquitin ligases that were increased in this model were

Cbl-b, Itch and GRAIL; all of which have been found to be involved in the negative regulation of T cell function (Chiang *et al.*, 2000; Fang *et al.*, 2002; Anandasabapathy *et al.*, 2003).

However, in adaptive tolerance the Ca^{2+}/calcineurin/NFAT pathway is inhibited (Anderson *et al.*, 2006; Chiodetti *et al.*, 2006) indicating that NFAT does not have a role in the maintenance of the tolerant state. Thus the transcriptional mechanisms that are involved in the maintenance of the hyporesponsiveness of tolerant T cells *in vivo* are unknown. Recent work from our group and from other laboratories has identified the transcription factor Early Growth Response 2 (Egr-2) as a potential candidate for maintaining tolerance (Harris *et al.*, 2004; Safford *et al.*, 2005; Anderson *et al.*, 2006).

1.3 Early Growth Response transcription factors

The Egr family of proteins consists of 4 members; Egr-1 (also called NGFI-A, Zif268, Znf225 or Krox-24), Egr-2 (also called Krox-20), Egr-3 (also called PILOT) and Egr-4 (also called NGFI-C). Egr proteins are rapidly induced in many cell types in response to stimulation. Indeed, Egr-2 was first identified as a gene that was induced in quiescent fibroblasts after serum stimulation (Chavrier *et al.*, 1988). Egr proteins are zinc finger transcription factors which contain 3 C2H2 type zinc finger motifs. Each zinc finger consists of 2 Cysteine and 2 Histidine residues co-ordinating a zinc ion (see Figure 1.3). The co-ordination of the zinc ion is important for structural stability since the motif has only limited hydrophobic interactions (Luisi, 1992). Structurally, the zinc finger motif consists of two anti-parallel β strands and an α helix (Pavletich and Pabo, 1991) (see also Figure 1.3). The α helix makes contacts to DNA base pairs in the major groove. Each zinc finger motif makes contacts with 3 base pairs (bp) of DNA; therefore the DNA binding sequence for Egr proteins is 9 bp in length.

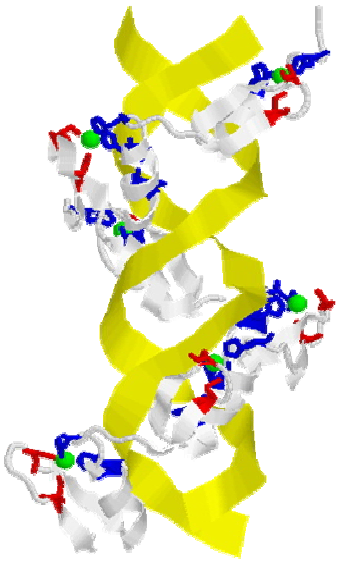

Figure 1.3: The zinc finger domain of Egr-1. 2 Cys (red) and 2 His (blue) co-ordinate a zinc ion (green). Data downloaded from Protein data bank (PDB ID: 1zaa) (Pavletich and Pabo, 1991) and displayed using Rasmol.

In addition to their DNA binding domains, Egr proteins have both transactivation and repressor domains, and so they can function both as transcriptional activators and repressors (see Thiel and Cibelli, 2002). Egr proteins have often been found to co-operate with other transcription factors in regulating target gene expression. For

example, Egr-1 was reported to synergise with NFAT leading to an increase in NFAT transcriptional transactivation of an IL-2 reporter construct while Egr-1 alone had little effect (Decker *et al.*, 1998). For some transcription factors, Egr proteins have been reported to both enhance and antagonise target gene expression. Egr-1 has been reported to co-operate with the zinc finger transcription factor SP-1 to increase expression of IL-2 receptor β (Lin and Leonard, 1997) but also to interfere with the activity of the same transcription factor by binding to overlapping SP-1 Egr protein binding sites and inhibiting SP-1 protein binding (Huang *et al.*, 1997). These two examples used different cell types and looked at different genes but serve to illustrate the complexity of transcriptional networks.

1.3.1 DNA binding specificity of Egr proteins

Egr-2 was first reported to bind to the sequence GCGGGGGCG (Chavrier *et al.*, 1990). An *in vitro* study, in which random PCR based mutagenesis of the Egr binding site was coupled to affinity based selection, identified the sequence GCGTGGGCG as the optimal binding site for Egr-2 (Swirnoff and Milbrandt, 1995). This is often described as the Egr-2 “consensus” site. The structural basis for this preference is not known. So far, Egr-1 is the only one of the Egr proteins for which the crystal structure of the DNA binding domain has been solved. The crystal structure of the DNA binding domain of Egr-1 bound to DNA (see Figure 1.3) revealed 11 important hydrogen bonds between amino acids in the Egr-1 protein and the G bases of residues at the 1, 3, 5, 6, 7, and 9 positions in the oligonucleotide (Pavletich and Pabo, 1991). The DNA binding domains of the Egr proteins are highly conserved, with approximately 90% identity, suggesting that the other Egr proteins bind to DNA in a similar fashion. Indeed, in the random PCR and affinity based selection study described above all the Egr proteins showed a

preference for G at all of these positions suggesting that these contacts are functionally important (Swirnoff and Milbrandt, 1995). Although many studies have reported Egr-2 binding to non-canonical binding sites, some of which differ considerably from the "consensus", most experimentally validated binding sites for Egr-2 also maintain this pattern of G residues (e.g. Mittelstadt and Ashwell, 1998;1999). However, notably in one of the more recently identified sites the G residue at the 1 position is not conserved (Laslo *et al.*, 2006). As more Egr-2 binding sites are found and experimentally validated it should be possible to determine the relative importance of each residue in the "consensus" binding site.

1.3.2 Egr-2 in the nervous system

Egr-2 has been extensively studied in the nervous system. Egr-2 KO mice die soon after birth due to defects in brain development; rhombomeres r3 and r5 fail to form properly demonstrating a critical role for Egr-2 in brain development (Schneider-Maunoury *et al.*, 1993). In addition, there are defects in peripheral nerve myelination in Egr-2 KO mice indicating that Egr-2 is essential for terminal Schwann cell differentiation (Topilko *et al.*, 1994). Indeed, Egr-2 has been proposed to be a master regulator gene of Schwann cell differentiation since it can induce expression of myelin genes even in fibroblasts (Parkinson *et al.*, 2004). Ectopic expression of Egr-2 in fibroblasts also inhibits stimuli-induced proliferation and confers protection from apoptosis (Parkinson *et al.*, 2004). Notably these phenotypes are similar to that of tolerant T cells; cell cycle arrested cells that remain alive for extended periods. Preliminary work by our group and others indicated that Egr-2 was expressed in tolerant T cells (Harris *et al.*, 2004; Safford *et al.*, 2005; Anderson *et al.*, 2006) suggesting the intriguing possibility that Egr-2 may have similar functions in T cells.

1.3.3 Egr family proteins in T cells

Egr-1, Egr-2 and Egr-3 are all expressed in DN thymocytes after the β-selection checkpoint correlating with pre-TCR signalling (Carleton *et al.*, 2002). They are also expressed both by DP thymocytes and mature T cells upon TCR stimulation (Shao *et al.*, 1997; Harris *et al.*, 2004; Safford *et al.*, 2005; Anderson *et al.*, 2006). Although the expression of Egr family proteins in response to pre-TCR and TCR signalling is well documented the roles of these proteins in the cell, and particularly the mechanisms of action, are less clear.

1.3.3.1 Egr proteins in thymocyte β-selection

In DN cells, Egr proteins are involved in the traversal of the β-selection checkpoint. Transgenic over-expression of Egr proteins in RAG-$1^{-/-}$ or RAG-$2^{-/-}$ thymocytes, which are arrested at the DN3 stage due to a lack of pre-TCR signalling, enables the cells to pass through the β-selection checkpoint to the DN4 and ISP stages (Miyazaki, 1997; Xi and Kersh, 2004). Conversely, dominant negative Egr-1 proteins can interfere with the development of DN cells to the DP stage (Carleton *et al.*, 2002). Thus Egr proteins are involved in thymocyte β-selection. However, there appears to be a degree of functional redundancy amongst the Egr proteins at this stage. Egr-1 KO mice have an increase in the total number of thymocytes but have normal percentages of DN thymocyte subsets and do not appear to have any defects in β-selection (Bettini *et al.*, 2002). Thymocytes from Egr-3 KO mice on the B6.AKR background do have decreased numbers of DN4 cells but this was attributed to decreased proliferation after β-selection rather than a defect in differentiation (Xi and Kersh, 2004). Similarly, Egr-1, Egr-3 double KO (DKO) mice on the C57BL/6 background have a decrease in the

number of DN4 thymocytes and decreased proliferation of DN4 cells (Carter *et al.*, 2007). This study also found increased apoptosis of DN4 cells which was not reported for Egr-3 KO mice on the B6.AKR background. Taken together these studies show that, while Egr proteins are involved in thymocyte β-selection, differentiation through the β-selection checkpoint occurs in the absence of individual Egr proteins suggesting that there is redundancy amongst the Egr proteins.

1.3.3.2 Egr proteins in thymocyte positive selection

Studies of Egr-1 KO and Tg mice revealed a role for Egr-1 in thymocyte positive, but not negative, selection (Miyazaki and Lemonnier, 1998; Bettini *et al.*, 2002). Both Egr-1 KO mice and TCR transgenic Egr-1 KO mice have decreased numbers of positively selected $CD4^+$ and $CD8^+$ SP thymocytes while clonal deletion proceeded normally (Bettini *et al.*, 2002). Conversely, expression of an Egr-1 transgene in TCR transgenic T cells allows their selection on normally non-selecting backgrounds (Miyazaki and Lemonnier, 1998). No selection was observed in mice lacking all MHC molecules indicating that enhanced Egr-1 expression on its own is not sufficient (Miyazaki and Lemonnier, 1998).

1.3.3.3 Mechanisms of Egr function in thymocytes

While roles for Egr proteins in thymocyte development are now well established, the mechanisms by which Egr proteins function in thymocytes are not as clear. Egr proteins have been reported to induce expression of the helix-loop-helix protein Id3 in thymocytes (Bain *et al.*, 2001; Xi *et al.*, 2006). Id3 is an inhibitor of E protein transcription factors which are required for thymocyte development (see Murre, 2005).

Id3 binds to E proteins and inhibits the binding of E proteins to DNA (see Murre, 2005). Ectopic expression of Egr-1 (Bain *et al.*, 2001) or Egr-3 (Xi *et al.*, 2006) in a murine DP thymocyte cell line, termed 16610D9, resulted in increased Id3 mRNA. Similarly, primary thymocytes from Egr-3 transgenic (Tg) mice have elevated Id3 mRNA and protein levels (Xi *et al.*, 2006). However, Egr-1, Egr-3 DKO mice on the C57BL/6 background reportedly have normal expression of Id3 (Carter *et al.*, 2007) calling into question the importance of Egr proteins in inducing Id3 expression under physiological conditions. As noted above, this study observed increased apoptosis of DN4 cells suggesting that supporting the survival of cells after β-selection may be an important function of Egr proteins. However, there were no obvious target genes that were altered in this study that would explain this effect. Instead, the authors reported a decrease in the expression of several metabolic enzymes suggesting that Egr proteins may serve to support survival indirectly via modulation of metabolism (Carter *et al.*, 2007). In addition, the authors found defective expression of the transcription factors Myc, and to a lesser extent, E2F1 and Runx, in DN3 cells from Egr-1, Egr-3 DKO mice and identified potential Egr binding sites in the promoters of these genes suggesting that this effect of Egr proteins may be secondary to induction of other transcription factors (Carter *et al.*, 2007). Hence a clearly defined mechanism of action for Egr proteins in thymocyte development is currently lacking.

1.3.3.4 Egr proteins in mature T cells

Egr-1 has been reported to be involved in T cell proliferation. Antisense oligonucleotides against Egr-1 mRNA partially inhibited the proliferative response of Rat lymphocytes induced by IL-2, phorbol esters or lectins (Perez-Castillo *et al.*, 1993). Subsequently, Egr-1 was reported to be involved in the expression of IL-2 (Decker *et*

al., 1998) and IL-2 receptor β (Lin and Leonard, 1997), providing a potential explanation for the decreased proliferation. However, lymphocytes from Egr-1 KO mice proliferate normally in response to lectin or anti-CD3 stimulation *in vitro* (Lee *et al.*, 1995). Similarly, purified $CD8^+$ cells from Egr-1 KO mice respond normally to anti-CD3 stimulation *in vitro* (Singh *et al.*, 2004). Furthermore, this study found increased levels of IL-2 production by Egr-1 KO T cells. These data indicate that Egr-1 is not required for T cell proliferation or, at least for $CD8^+$ cells, for IL-2 expression after TCR stimulation.

1.3.3.5 Egr-2 and Egr-3 and FasL

Fas is a TNF receptor family death receptor that is induced on T cells after TCR stimulation. Fas ligand (FasL) is a membrane associated cytokine of the TNF family that is also induced after TCR engagement and can bind to the Fas receptor. Ligation of the Fas receptor with its ligand induces a signalling cascade that results in the induction of apoptotic cell death (see Siegel *et al.*, 2000). Both Fas and FasL are expressed after TCR stimulation and this pathway plays an important role in the deletion of effector T cells at the end of an immune response. Egr-2 and Egr-3 have been reported to be involved in FasL induction in response to TCR stimulation (Mittelstadt and Ashwell, 1998;1999; Rengarajan *et al.*, 2000). This group found that Egr-2 and Egr-3 bound to a sequence from the FasL promoter *in vitro*, that they termed the FasL Response Element (FLRE). Although at first glance the minimal FLRE site (5'-GTGGGTGT-3') appears to be substantially different from the Egr-2 "consensus" site, in the FasL promoter it is flanked by 5'-GA-3' at its 5' end which would maintain the pattern of G residues that appear to be functionally important in Egr protein binding (see above). Over-expression of Egr-2 or Egr-3 could drive expression of a FasL reporter construct in both T cell

hybridomas and non-lymphoid cell lines (Mittelstadt and Ashwell, 1998;1999), indicating that Egr-2 and Egr-3 have a role in FasL expression.

1.3.3.6 Signalling pathways controlling Egr-2 expression

Several studies have implicated NFAT in the induction of Egr-2 expression following TCR engagement. Cyclosporine treatment, which inhibits calcineurin and hence NFAT activation, partially inhibits Egr-2 expression (Mittelstadt and Ashwell, 1999). Furthermore, $CD4^+$ T cells from NFAT1, NFAT4 double KO mice have a defect in Egr-2 and Egr-3, but not Egr-1, expression following TCR stimulation while NFAT proteins can drive expression of Egr-2 and Egr-3 promoter reporter constructs in hybridoma or lymphoma cell lines (Rengarajan *et al.*, 2000). However, there was still low but detectable Egr-2 expression in both cyclosporine treated and NFAT1, NFAT4 deficient cells following TCR engagement (Mittelstadt and Ashwell, 1999; Rengarajan *et al.*, 2000). In addition, tolerant T cells have impaired induction of NFAT activity following TCR stimulation but still strongly induce Egr-2 expression (Anderson *et al.*, 2006). These data suggest that, while NFAT is important for induction of Egr-2, there are additional pathways that can regulate Egr-2 expression in T cells.

1.3.3.7 Egr-2 in tolerance and anergy

While we were working on this project two other groups published their initial findings on the role of Egr-2 in tolerance and anergy. One group used RNAi technology to "knockdown" Egr-2 expression in an established T cell line which partially inhibited the induction of clonal anergy by anti-CD3 treatment (Harris *et al.*, 2004). Egr-2 "knockdown" also increased the response of pre-activated cells to restimulation with

anti-CD3 and anti-CD28 indicating that the function of Egr-2 was not limited to clonal anergy (Harris *et al.*, 2004). Another group took an alternative approach using a lentiviral system to over-express Egr-2 and this treatment resulted in reduced production of IL-2 after T cell activation *in vitro* (Safford *et al.*, 2005). The latter group also observed slightly elevated levels of the E3 Ubiquitin ligase Cbl-b in Egr-2 transduced T cells and proposed the hypothesis that Egr-2 regulates TCR signalling through induction of E3 Ubiquitin ligases (Safford *et al.*, 2005). Given that NFAT has been reported to induce E3 Ubiquitin ligases in clonal anergy and given the data supporting a role for NFAT in Egr-2 expression, this hypothesis is thus analogous to that proposed for NFAT and Egr-2 and Egr-3 in FasL expression. However, these hypotheses have not been tested *in vivo* since Egr-2 knockout mice die perinatally due to defects in hindbrain development (Schneider-Maunoury *et al.*, 1993). Thus, the functions of physiological levels of Egr-2 in T cells *in vivo* have not been studied. To assess the functions of Egr-2 in T cells *in vivo*, we established Egr-2 conditional knockouts (Egr-2 cKO), in which the Egr-2 gene was deleted specifically in $CD2^{+}$ lymphocytes, using the Cre loxP system.

1.4 Cre loxP system of tissue specific KO mice

The Cre loxP system can be used to generate tissue specific knockout mice (reviewed in Lewandoski, 2001) (see also Figure 1.4). This system uses the Cre recombinase from bacteriophage P1. Cre recognises 34bp DNA sequences termed loxP sites and catalyses recombination between them. If 2 loxP sites are arranged in the same orientation, this results in the deletion of the intervening DNA. Generation of tissue specific KO mice requires the establishment of two genetically modified mouse lines; one where loxP sites are introduced in the gene of interest and another where the expression of the Cre

transgene is restricted to a particular tissue. To generate the first line, loxP sites are introduced into introns of the gene of interest and this construct is used to replace the endogenous gene in embryonic stem (ES) cells by homologous recombination. ES cells where this has occurred are selected and then injected into the blastocoele of a mouse embryo where they can contribute to the tissues of the resulting mouse. If the ES cell contributes to the germline of the resulting animal the mouse will transmit the targeted gene to some of its progeny. These progeny which possess the targeted gene are selected and bred to homozygosity to obtain a loxP/loxP line. This line is then crossed with a line that expresses Cre under the control of a tissue specific promoter. This second line can be generated by cloning the Cre gene downstream of a tissue specific promoter and then introducing this construct into fertilised ova. In some of the ova the construct will integrate into the host genome; the mice that derive from these ova are identified by genotyping and serve as founders for tissue specific Cre lines. These two lines are then crossed to generate loxP/loxP Cre transgenic mice. Expression of Cre under the control of a tissue specific promoter results in deletion of the targeted gene occurring only in that tissue.

Egr-$2^{loxP/loxP}$ mice have two loxP sites flanking exon 2 of the Egr-2 gene which encodes the majority of the protein including the DNA binding domain (Taillebourg *et al.*, 2002). hCD2-Cre mice have expression of Cre driven by the promoter and LCR of the human CD2 gene (de Boer *et al.*, 2003). These regulatory elements drive expression of the Cre transgene in lymphocytes in a copy number dependent and integration site independent fashion (de Boer *et al.*, 2003). This means that the expression of the Cre gene is dependent upon the number of copies in the genome irrespective of where the hCD2-Cre construct has integrated.

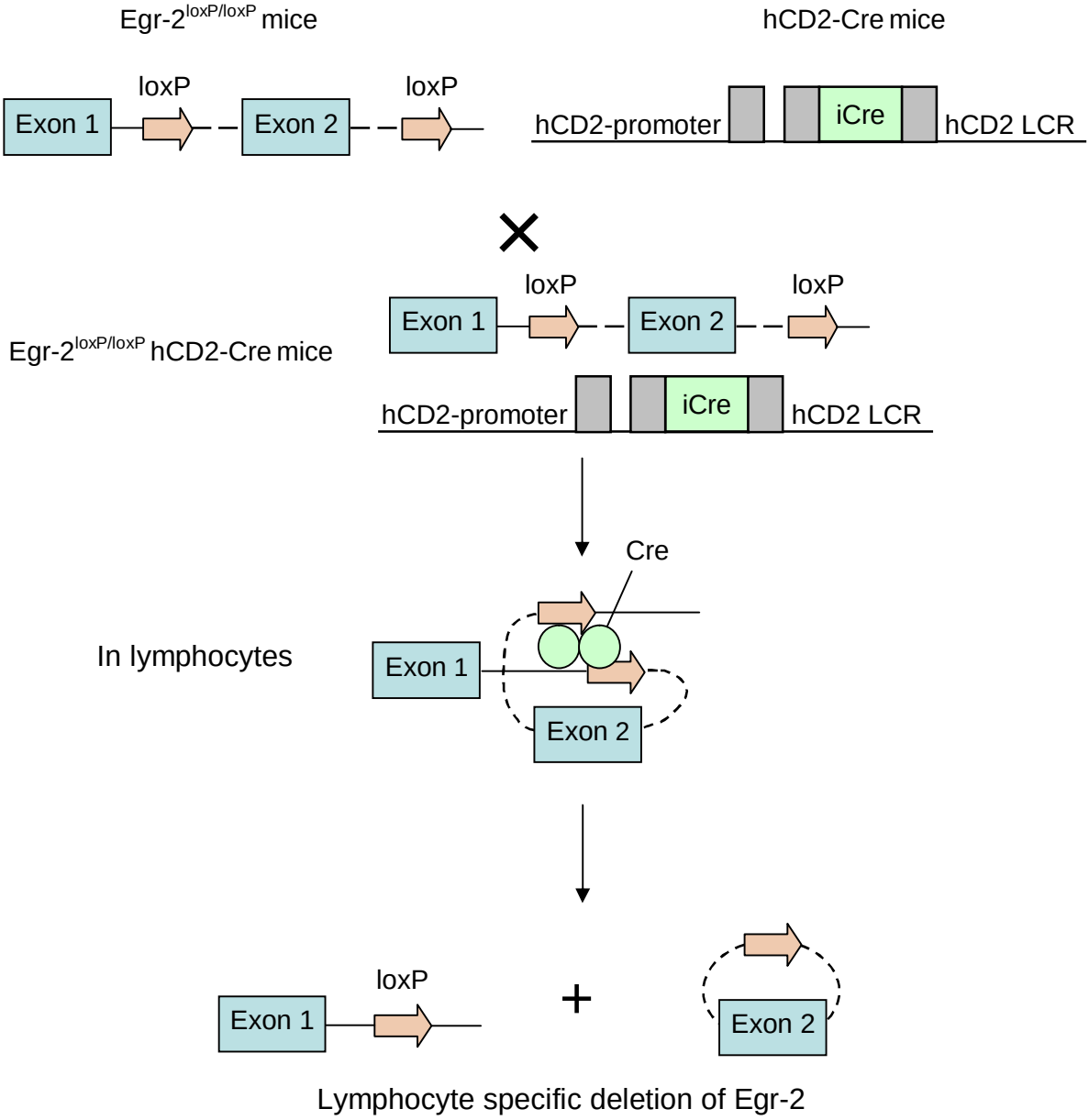

Figure 1.4: Cre loxP system of tissue specific KO mice. This system requires the generation of two mouse lines; one with two loxP sites introduced into introns of the target gene (Egr-2$^{loxP/loxP}$ mice, top left) and the other with the expression of Cre under the control of tissue specific regulatory regions (hCD2-Cre mice, top right); which are then interbred. The enzyme Cre recognises the loxP sites in the target gene and catalyses recombination between them resulting in the deletion of the intervening DNA (exon 2 of the Egr-2 gene). Limiting Cre expression to a specific tissue (lymphocytes), due to the use of the tissue specific regulatory regions (hCD2), results in the deletion of the target gene only occurring in that tissue; hence yielding tissue specific KO mice. See text for further details. Based on figures from (Lewandoski, 2001).

These two lines were crossed to generate Egr-2$^{loxP/loxP}$ hCD2-Cre mice which have deletion of Egr-2 only in lymphocytes. These mice should therefore be viable and permit the analysis of T cell populations. The aims of this project were to analyse the phenotype of these Egr-2$^{loxP/loxP}$ hCD2-Cre mice and to investigate the mechanisms of Egr-2 function.

Chapter 2: MATERIALS and METHODS

2.1 Tg4 Transgenic Mice

Tg4 transgenic mice, a TCR Tg line where the $CD4^+$ T cells bear a TCR with affinity for the acetylated N-terminal peptide of MBP Ac1-9 (Liu *et al.*, 1995), on the B10.PL background (Anderson *et al.*, 2006) were maintained at the University of Bristol. Tolerance was induced in Tg4 transgenic mice by ten intranasal (i.n.) administrations of 25µl of a 4mg/ml solution of the high affinity Ac1-9 analogue, Ac1–9[4Y], at 3-4 day intervals as described (Anderson *et al.*, 2006). Total splenocytes were extracted from Tg4 mice before (A0) and 2 hours after (A2) the first i.n. Ac1-9[4Y] peptide administration and before (T0) and after (T2) the tenth treatment as described (Anderson *et al.*, 2006). Total RNA and protein were then isolated at the University of Bristol by Professor D. Wraith and colleagues and were given to us for analysis.

2.2 Egr-2 conditional knockout mice

Egr-$2^{loxP/loxP}$ mice and hCD2-Cre mice were kind gifts from Dr P. Charnay, Institut National de la Santé et de la Recherche Médicale, Ecole Normale Supérieure, Paris, and Dr D. Kioussis, National Institute for Medical Research, London, respectively (Taillebourg *et al.*, 2002; de Boer *et al.*, 2003). Egr-2 conditional knockout mice (Egr-2 cKO) were generated by crossing Egr-$2^{loxP/loxP}$ mice with hCD2-Cre transgenic mice. All Egr-2 cKO mice used in this study were backcrossed at least three times to C57BL/6. All Egr-2 cKO mice used in this study were aged 3-4 months unless otherwise stated. Egr-2 cKO mice were maintained in the Biological Services Unit,

Barts and The London School of Medicine, and used according to established institutional guidelines under the authority of a UK Home Office project licence (Guidance on the Operation of Animals, Scientific Procedures Act 1986).

2.3 hCD2 Egr-2 transgenic mice

To generate hCD2 Egr-2 transgenic mice, the Egr-2 gene was first cloned by PCR amplification of the Egr-2 gene in the pBABE-Egr-2 EGFP plasmid (a kind gift from Professor K. Jessen, University College London) using primers sense 5'-TGACGAATTCATGATGACCGCCAAGGC-3' and antisense 5'-CACACCCTAACTGACACACATTCC-3' and the Phusion High Fidelity PCR kit (New England Biolabs) by Dr B. Zhu as per the manufacturer's instructions. The sense primer contains an EcoRI site towards the 5' end while the location of the antisense primer was chosen so that an EcoRI site present in the pBABE-Egr-2 GFP plasmid was also present in the PCR product. The PCR product and the hCD2 VA vector (a kind gift from Dr D. Kioussis, National Institute for Medical Research, London) (Zhumabekov *et al.*, 1995) were both digested with EcoRI (New England Biolabs) by Dr B. Zhu according to the manufacturer's instructions. The vector and the PCR product were then mixed together with T4 DNA ligase (New England Biolabs) by Dr B. Zhu as per the manufacturer's instructions. The ligation reaction was then used to transform DH10α *Escherichia coli* bacteria by heat shock by Dr B. Zhu. Briefly, the ligation reaction was added to 60µl of DH10α bacteria, the sample heated to 42°C for 2 minutes and then placed on ice for 2 minutes followed by incubation at 37°C for 30 minutes in lysogeny broth (LB) medium before transfer to agar plates. Four clones were selected and the orientation of the insert in the construct was analysed by sequential restriction digestion with SmaI (25°C) and then BamHI (37°C) (both New England Biolabs) by Dr B. Zhu

according to the manufacturer's instructions. Plasmids were isolated from the clones with the correct orientation by mini-prep using the QIAquick spin mini-prep kit (Qiagen) and then digested with NotI and SalI (New England Biolabs) using the manufacturer's protocols by Dr B. Zhu. The resulting mixture was separated by electrophoresis and a band of 14.2kb in size, representing the hCD2 Egr-2 construct, was extracted from the gel and purified using the Geneclean turbo kit (Qbiogene) according to the manufacturer's protocol by Dr B. Zhu. The hCD2 construct was then injected into pro-nuclei of fertilised oocytes of a C57BL/6 × CD1 background by Mr W. Mansfield of the Transgenic core facility, Queen Mary, University of London. Transgenic mice were identified as below and two founder lines, Tg14 and Tg18, were established by backcrossing at least three times to C57BL/6. All hCD2-Egr-2 Tg mice used in this study were aged 3-4 months unless otherwise stated. Egr-2 Tg mice were maintained in the Biological Services Unit, Barts and The London School of Medicine, and used according to established institutional guidelines under the authority of a UK Home Office project licence (Guidance on the Operation of Animals, Scientific Procedures Act 1986).

2.4 Genotyping

Mice tails were collected and genomic DNA extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma) by Dr B. Zhu according to the manufacturer's instructions. Egr-$2^{loxP/loxP}$ hCD2-Cre mice and hCD2-Egr-2 Tg mice were identified by polymerase chain reaction (PCR), a technique that allows selective exponential amplification of DNA sequences. This procedure takes advantage of a thermostable DNA polymerase from *Thermus aquaticus* termed Taq. This enzyme is not denatured at temperatures that denature DNA and so it is possible to perform several rounds of extension without

adding new DNA polymerase enzyme each cycle. Since DNA synthesis must be primed, the use of primers specific for a particular gene allows this technique to selectively amplify that locus. Taq extends the primers by incorporating dNTPs complementary to the pre-existing strand thus producing a copy of the DNA. PCR was performed as per the manufacturer's protocol with a final primer concentration of 0.5µM. Genotyping primer sequences are shown in Table 2.1. Egr-2 locus primer sequences were provided by Dr P. Charnay, Institut National de la Santé et de la Recherche Médicale, Ecole Normale Supérieure, Paris. hCD2-Cre primers were designed to amplify a 133bp fragment of the codon optimised Cre recombinase gene (Genbank Accession number: AY056050); a version of Cre that has been altered via synonymous mutations to better reflect mammalian codon usage (Shimshek *et al.*, 2002). hCD2-Egr-2 construct primers were designed so that the sense primer annealed to exon 1 of the human CD2 gene while the antisense primer annealed to a region just upstream of the Egr-2 transcriptional start site.

Primer		Sequence
Egr-2 locus	sense	5'-AGTTGACAGCCCGAGTCCAGTGG-3'
	antisense	5'-GGGAGCGAAGCTACTCGGATACGG-3'
hCD2-Cre	sense	5'-CCAACAACTACCTGTTCTGCCG-3'
	antisense	5'-TCATCCTTGGCACCATAGATCAGG-3'
hCD2-Egr-2 construct	sense	5'-CCACCAGTCTCACTTCAGTTCC-3'
	antisense	5'-CAGCTGGTGCATAAAACCACTG-3'

Table 2.1: Genotyping primer sequences.

2.5 Isolation of CD4$^+$ cells

Lymphoid organ extraction was performed by Dr B. Zhu. When using splenocytes, erythrocytes were lysed using the ACK Lysing Buffer (Lonza) according to the manufacturer's instructions. This buffer uses a hypotonic solution to lyse the

erythrocytes while maintaining the viability of the lymphocytes. $CD4^+$ cells were then isolated by Magnetic Activated Cell Sorting (MACS) using a positive selection kit (Miltenyi) according to the manufacturer's instructions. Total splenocytes were incubated with anti-CD4 antibodies attached to magnetic beads at 4°C for 30 minutes in Phosphate Buffered Saline (PBS) containing 3% Foetal Bovine Serum (FBS). This labels any $CD4^+$ cells with magnetic beads. The cells were then washed and run through a magnetic column. $CD4^+$ cells are retained on the column due to the magnetic beads while the unlabelled cells pass through the column. The column was washed three times with PBS containing 3% FBS to remove any non-specifically bound cells and then the $CD4^+$ cells were eluted by removing the column from the magnetic field and washing with PBS containing 3% FBS. $B220^+$ cells were also isolated by MACS using a positive selection kit (Miltenyi) by Dr S. Li at Brunel University (Zhu *et al.*, 2008).

2.6 Cell culture and stimulation

T cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 300mg/L L-glutamine supplemented with 10% FBS, 50μM 2-mercaptoethanol (2-ME), and 50 units/ml penicillin and 50μg/ml streptomycin (all from Invitrogen). $CD4^+$ T cells, and MF2.2D9 cells, were stimulated with beads coated with 1μg/ml anti-CD3ε antibody (clone 145-2C11; BD Biosciences) and 1μg/ml anti-CD28 antibody (clone 37.51; BD Biosciences), or plates coated with 5μg (or different amounts as indicated) anti-CD3ε antibody with or without 2.5μg/ml of soluble anti-CD28 antibody as indicated. $B220^+$ cells were stimulated with anti-surface Ig antibody for 6 hours by Dr S. Li at Brunel University (Zhu *et al.*, 2008).

2.7 RNA Extraction

Total RNA was extracted using the Trizol reagent (Invitrogen). This method is an adaptation of the Phenol Chloroform extraction method to separate nucleic acids from other cellular components. This protocol uses acidic conditions which results in the separation of the RNA from the DNA and proteins following Phenol Chloroform extraction. The solution also contains Guanidinium thiocyanate; a strong denaturing agent which denatures any proteins, such as RNAse, present in the sample. RNA extraction was performed according to the manufacturer's protocol. Briefly, cells were pelleted and then lysed in 1ml of Trizol per 10^7 cells and then incubated at room temperature for 10 minutes. 200µl of Chloroform was then added, the sample mixed vigorously, incubated at room temperature for 5 minutes and then centrifuged. After centrifugation the sample separated into a red organic phase and a clear aqueous phase separated by a white interphase. The RNA separated into the aqueous phase while proteins and DNA remained in the organic phase or interphase. The aqueous phase was transferred to a new tube and the RNA was precipitated by the addition of 2 volumes of 100% ethanol. Nucleic acids are not soluble in alcohol and so the RNA precipitates out of solution. In addition, in situations where the initial cell number was low, glycogen was added; glycogen is also not soluble in alcohol and co-precipitates with the RNA aiding visualisation of the RNA pellet. The RNA was incubated at −20°C for 1 hour and then spun down. After centrifugation the RNA forms a pellet at the bottom of the tube. The pellet was washed with 70% ethanol and then resuspended in RNAse-free water.

2.8 Reverse Transcription

RNA was converted to first strand cDNA using a reverse transcriptase enzyme from Murine Moloney Leukaemia Virus (MMLV-RT) (Promega). This enzyme catalyses the formation of a complementary cDNA strand using the RNA strand as a template. Oligo-dT nucleotides anneal to the poly-A tails of mRNA and these oligo-dT nucleotides act as primers for reverse transcriptase. The amount of nucleic acid present in these samples was detected by measuring the absorbance at 260nm on a nanodrop spectrophotometer. Volumes of solutions were chosen to give 2µg RNA per reaction and 0.5µg of oligo-dT was added to each tube. These were then heated to 70°C for 10 minutes to denature any secondary structures present in the RNA, facilitating the binding of the oligo-dT primers. The samples were then placed on ice. Reagents were added to give a final concentration of 500µM of each of dATP, dTTP, dCTP and dGTP, 1 Unit/µl of RNAse Inhibitor and 10 Units/µl of MMLV-RT in 1x RT buffer (all Promega). The reactions were then heated to 37°C for 1 hour and then to 70°C for 10 minutes to inactivate the MMLV-RT enzyme. 80µl of ddH_20 was then added to each sample to give a final volume of 100µl and the samples were then stored at −20°C.

2.9 Real Time RT-PCR

Real Time PCR uses the same principle as normal PCR described above but allows the quantitative comparison of samples. A molecule which fluoresces when intercalated into dsDNA, called SYBR Green, was added to the reaction mixture. After each round of product synthesis the amount of product is calculated by measuring the fluorescence. Real Time PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) as per the manufacturer's instructions using 1µl of relevant cDNA and a final

concentration of 0.2μM of relevant sense and antisense primers in 1x SYBR Green Master Mix. Real Time PCR was performed on a Rotor-Gene system (Corbett Robotics) using the program listed below:

1. 95°C for 10min
2. 95°C for 20s
3. 58°C for 30s
4. 72°C for 20s

2-4 40 cycles

The initial 95°C step, in addition to denaturing the DNA, also denatures the antibody that is bound to the DNA polymerase allowing the enzyme to catalyse DNA synthesis during the elongation phase. During each elongation phase fluorescence data was collected. After the cycles had finished a melting curve analysis was performed in which the fluorescence was continually monitored as the temperature increased from 50°C to 99°C. When the temperature reaches the melting temperature (Tm) for each DNA molecule 50% of that molecule is denatured which is detectable as a decrease in fluorescence. This can be used to calculate the Tm of each product allowing detection of non-specific products such as primer dimers. In addition, once the PCR reaction had completed the products were routinely run on an agarose gel to confirm that there was only one band of the correct size.

2.9.1 Primer design

For Real Time PCR, primers were designed to have approximately 20 nucleotides with around 50% G/C content. It is important that the 3' end binds stably to the cDNA to

allow the polymerase to extend the chain; hence the last 6 nucleotides at the 3' end were designed to contain at least 3 G/Cs. The sense and antisense primers were designed so that they anneal to different exons of the sequence of interest. This allows identification of genomic DNA contaminants as these will contain the intervening introns in addition to the exons so will be larger and thus detectable via electrophoresis. The Tm of each primer was estimated using the Wallace rule (Tm = 4 × [G + C] + 2 × [A + T]) and the annealing temperature was initially set to Tm − 2°C and altered as necessary. Real Time PCR primer sequences are shown in Table 2.2.

Primer		Sequence
Egr-2	sense	5'-CTTCAGCCGAAGTGACCACC-3'
	antisense	5'-GCTCTTCCGTTCCTTCTGCC-3'
p21cip1	sense	5'-TTGCACTCTGGTGTCTGAGC-3'
	antisense	5'-GAGGACCAATCTGGGCTTGG-3'
IFN-γ	sense	5'-CCATCAGCAACAACATAAGC-3'
	antisense	5'-AGCTCATTGAATGCTTGGCG-3'
IL-2	sense	5'-GCATGTTCTGGATTTGACTC-3'
	antisense	5'-CAGTTGCTGACTCATCATCG-3'
IL-17A	sense	5'-AGCGTGTCCAAACACTGAGG-3'
	antisense	5'-CTATCAGGGTCTTCATTGCG-3'
IL-17F	sense	5'-AACCAGGGCATTTCTGTCCC-3'
	antisense	5'-TTTCTTGCTGAATGGCGACG-3'
IL-1α	sense	5'-ATGTATGCCTACTCGTCGGG-3'
	antisense	5'-ATGAGGTCGGTCTCACTACC-3'
CCR6	sense	5'-GACTGGAGCTGTTCTTTGGG-3'
	antisense	5'-CACTTTGCCCGTGTTGACCG-3'
IL-21	sense	5'-CTCAAGCCATCAAACCCTGG-3'
	antisense	5'-CATACGAATCACAGGAAGGG-3'
IL-10	sense	5'-GGTTGCCAAGCCTTATCGGA-3'
	antisense	5'-ACCTGCTCCACTGCCTTGCT-3'
IL-15	sense	5'-AGAAACGTGCTCTACCTTGC-3'
	antisense	5'-GATGAACATTTGGACAATGCG-3'
IL-4	sense	5'-CAAACGTCCTCACAGCAACG-3'
	antisense	5'-CTTGGACTCATTCATGGTGC-3'
IL-13	sense	5'-ATTCCCTGACCAACATCTCC-3'
	antisense	5'-GTTGCTTTGTGTAGCTGAGC-3'
Bcl-2	sense	5'-CGTCAACAGGGAGATGTCACC-3'
	antisense	5'-GGAGAAATCAAACAGAGGTCGC-3'
β-actin	sense	5'-AATCGTGCGTGACATCAAAG-3'
	antisense	5'-ATGCCACAGGATTCCATACC-3'

Table 2.2: Real Time PCR primer sequences.

2.9.2 Data analysis

The data were analysed using the Rotor-Gene Software. In each reaction there is an exponential phase, when the number of copies of the DNA molecule doubles (at least theoretically) during each cycle, and a plateau phase when the amount of one of the reagents becomes limiting and the increase is small. Analysis was performed by choosing a threshold value which is still in the exponential phase and calculating how many cycles (Ct) it takes for the florescence to reach the threshold for each sample. These Ct values were then compared to the Ct values for a reference gene the expression of which does not change significantly; this normalisation corrects for small variances in mRNA amount and permits the comparison of different samples. β-actin was used as a reference gene and relative expression was calculated by the ΔΔCt method using: relative expression=$2^{(Ct_{(\beta\text{-actin})}-Ct_{(target)})}\times 10{,}000$. All samples were run in duplicate.

2.10 Protein extraction

To obtain protein extracts, the cells were swelled on ice for 15 minutes in a hypotonic buffer containing 10mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid], 10mM KCl, 0.1mM EDTA [ethylenediaminetetraacetic acid], 0.2mM DTT [dithiothreitol], 0.5mM PMSF [phenylmethylsulphonyl fluoride] and protease and phosphatase inhibitors (Roche). Then the detergent Igepal CA-630 (Sigma) was added to a final concentration of 1% and the samples mixed. This relatively mild detergent lyses the plasma membrane but does not break the nuclear membrane. The tubes were then centrifuged to pellet the nuclei. The supernatant containing the cytosolic extract was transferred to a new tube. The pellet containing the nuclei was resuspended in a

buffer containing 20mM HEPES, 0.4M NaCl, 1mM EDTA, 1mM EGTA [ethylene glycol tetraacetic acid], 1mM DTT, 1mM PMSF and protease and phosphatase inhibitors (Roche). The nuclei were vigorously agitated in this buffer for 10 minutes at room temperature and then spun down. Following centrifugation the nuclear proteins were located in the supernatant which was transferred to a new tube. This nuclear extract and the cytosolic extract were aliquoted and stored at −80°C.

2.11 SDS PAGE and Western blotting

SDS PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis) is a technique that separates proteins based on their size. The migration of a molecule through an electric field is dependent upon its charge and its size. Unlike DNA molecules where the charge is proportional to the size due to the similar nature of the monomers, there is no relationship between charge and size for proteins. To overcome this proteins are treated with the detergent SDS which results in coating of the proteins with negatively charged sulphate groups. This is because the hydrophobic dodecyl chains of SDS interact with hydrophobic amino acids in the proteins. The amount of hydrophobic residues is usually proportional to the size of the protein; hence the amount of associated SDS and consequently the degree of negative charge is also proportional to the size. Therefore, once in an electric field SDS treated proteins will migrate depending on their size and can be separated by electrophoresis using a Polyacrylamide gel.

Western blotting was performed as per the protocol from (Sambrook *et al.*, 1989) using a 10% resolving gel; composed of 10% acrylamide: 0.26% bisacrylamide solution (ProtoGel), 0.1% SDS, 0.1% APS [Ammonium Persulphate], 0.04% Temed [N,N,N',N'-

tetramethylethylene diamine] and 380mM Tris [tris(hydroxymethyl)aminomethane] pH8.8; and a 5% stacking gel; composed of 5% acrylamide: 0.13% bisacrylamide solution (ProtoGel), 0.1% SDS, 0.1% APS, 0.1% Temed and 187.5mM Tris pH6.8.

The protein samples were mixed with loading buffer containing SDS and 2-mercaptoethanol and then heated to 95°C for 5 minutes to denature and reduce the proteins. The protein samples, and 5µl of Rainbow molecular marker (Amersham), were loaded into the wells of the gel and a potential difference of 100V was applied for 1.5 hours. After separation by electrophoresis the proteins were transferred to a nitrocellulose membrane by a wet transfer protocol. The gel was placed on top of a nitrocellulose membrane (Amersham) and this was sandwiched between two pieces of filter paper. This was placed in a cassette in the wet transfer system and a current of 300mA applied for 1 hour. The negatively charged proteins move towards the anode but are unable to pass through the nitrocellulose membrane and hence are retained on this surface in the same orientation as in the gel.

To prevent antibody binding non-specifically, the membrane was blocked in 5% milk in TBST (Tris Buffered Saline with 0.1% Tween 20 [a detergent]) for 1 hour at room temperature. The membrane was then incubated overnight at 4°C with the relevant primary antibody against p21cip1 (Santa Cruz Biotechnology), Egr-2 (Covance), Histone H3 (Abcam) or β-actin (Sigma) in 5% milk in TBST at a 1:100 dilution (β-actin), 1:200 dilution (p21cip1 and Egr-2) or 1:1000 dilution (Histone H3). These antibodies were raised by immunising a rabbit with a peptide derived from the protein of interest. Therefore the antibody will bind to any protein containing this sequence present on the membrane. The following day any unbound primary antibody was removed by washing the membrane 3 times in TBST. The membrane was then

incubated with a polyclonal goat anti-rabbit IgG HRP (horseradish peroxidase) conjugated secondary antibody (Biorad) in 5% milk in TBST, at a 1:2000 dilution, for 1 hour at 4°C with rotation. This secondary antibody, raised in goat against rabbit antibody, is conjugated to the reporter enzyme HRP to enable detection. The membrane was then washed 3 times in TBST and once in TBS to remove any unbound secondary antibody and then transferred onto cling film. The immune complexes were detected by chemiluminescence using the ECL Plus kit (Amersham) according to the manufacturer's instructions. This reagent contains a substrate that is oxidised, catalysed by HRP, producing a luminescent signal. Following exposure to ECL Plus for 5 minutes, the luminescence was detected by exposing photographic film to the membrane and then developing the film. Analysis of FasL, pERK and total ERK expression by western blot was performed by Dr B. Zhu.

2.12 Proliferation assay

To analyse proliferation of T cells tritiated thymidine ([^{3}H]TdR) incorporation was measured by Dr B. Zhu. This technique takes advantage of the fact that as cells proliferate they replicate their DNA. This technique uses the nucleotide thymidine (TdR) that is labelled with a radioactive isotope of hydrogen called tritium (^{3}H). As the cells proliferate they take up this [^{3}H]TdR and incorporate it into their DNA. Thus tritium incorporation is proportional to DNA synthesis and hence to proliferation. To measure [^{3}H]TdR incorporation, purified $CD4^+$ T cells (5×10^4 cells/200µl) in 96-well plates were stimulated in triplicate for 72 hours. 1µCi of [^{3}H]TdR was added to each well for the last 8 hours of culture, then the DNA was harvested onto glass fibre filters and unincorporated [^{3}H]TdR was washed away. Tritium incorporation was measured by

scintillation counting; tritium emits β radiation which excites scintillation fluid causing it to emit light which can then be detected.

2.13 Flow cytometry

Flow cytometry permits the investigation of the expression of cellular markers by a mixed population of cells. This technique uses antibodies that have high affinity for the protein of interest. These antibodies are usually directly conjugated to a fluorescent marker to enable detection. Total splenocytes or thymocytes were incubated with the indicated fluorescently labelled antibodies on ice for 30-60 minutes in PBS containing 3% FBS. Flourescein isothiocyanate (FITC)-conjugated antibodies to CD3, CD4 and CD62L; phycoerythrin (PE)-conjugated antibodies to B220, CD4, CD8, CD25 and CD69; allophycocyanin (APC)-conjugated antibody to CD44 and peridinin chlorophyll protein (PerCP)-conjugated antibody to CD3 were all from BD Biosciences while APC-conjugated antibodies to NK1.1, CD5, CD11b and CD25 and PE-Cy5-conjugated antibody to TCR-β were all from eBioscience. The cells were then washed and resuspended in PBS and run through the flow cytometer. The cells are passed through a nozzle to create a stream of single cells. The machine uses lasers of particular wavelengths to excite the fluorochromes attached to the antibodies which then emit at a longer wavelength. PhotoMultiplier Tubes (PMT) detect these emissions for each cell that passes through the machine. The computer then plots this data so that the expression of the markers by the cells can be quantified. In addition the computer can be directed to show only those cells in a certain population; a process termed gating. In this study we gated on $CD4^+$ or $CD8^+$ populations to investigate the expression of various markers and on $CD4^-CD8^-$ cells to examine DN cell subpopulations.

To determine apoptosis induction, cells were stained with Annexin V; a protein that binds the phospholipid phosphatidylserine. This phospholipid is normally confined to the interior surface of the cell membrane but is externalised in apoptotic cells. Thus Annexin V stains cells undergoing apoptosis. The cells were also stained with Propidium Iodide (PI) to exclude those cells that were already dead; PI binds to nucleic acids but can only gain access to this material if the cell has lost membrane integrity, thus PI positive cells are dead cells. Staining was performed according to the protocol supplied by BD Biosciences. Briefly, cells were resuspended in a buffer containing 10mM Hepes (pH 7.4), 140mM NaCl, 2.5mM $CaCl_2$, and APC conjugated Annexin V and PI were added. The cells were incubated for 15 min at room temperature in the dark, then diluted in the same buffer and analysed by flow cytometry.

To examine cytokine expression we stimulated splenocytes with 20ng/ml Phorbol 12-myristate 13-acetate (PMA) and 0.5μg/ml Ionomycin for 5 hours in the presence of Brefeldin A, an inhibitor of cytokine secretion, and then stained for surface markers. We then performed intracellular cytokine staining using the Fixation & Permeabilisation Kit from eBioscience according to the manufacturer's protocol. Briefly, the cells were fixed with paraformaldehyde and then permeabilised using the relevant buffers in the Kit before incubation with FITC labelled antibody against IFN-γ, or PE labelled antibody against IL-4 or IL-2 (eBioscience). Cells were gated on the $CD4^+$ population. Analysis of intracellular IL-17 was performed by Dr S. Li at Brunel University (Zhu *et al.*, 2008).

To determine FoxP3 expression, we used the Foxp3 Staining Buffer Set from eBioscience according to the manufacturer's instructions. Briefly, cells were stained with surface markers and then fixed and permeabilised using the relevant buffers in the Set before incubation with PE labelled antibody against FoxP3 (clone FJK-16s; eBioscience). Cells were gated on the $CD4^+$ population.

Flow cytometry data was generated using a BD LSR II and initially analysed using the BD FACSDiva software. Where applicable, data was further analysed using FlowJo software (TreeStar Inc.).

2.14 Fluorescence Activated Cell Sorting (FACS)

Variants of flow cytometry machines can be used to sort cells based on the expression of markers. This technique is called Fluorescence Activated Cell Sorting (FACS). FACS is similar to normal flow cytometry but after the cells have passed the PMT and the expression of the markers has been analysed, a charge is placed upon the cell, if it expresses a given marker, which directs it down one side of a deflection screen thus separating it from cells that do not express that marker. In this study, $CD4^+$ $CD25^-CD44^{low}$ and $CD4^+CD25^-CD44^{high}$ cells were isolated by FACS from MACS enriched $CD4^+$ populations. In addition, retrovirally transduced Enhanced Green Fluorescent Protein (EGFP) positive cells (see section 2.17) were isolated from non-transduced $EGFP^-$ cells.

2.15 In vivo proliferation

To analyse the proliferation of T cells *in vivo*, the BrdU flow kit from BD Biosciences was used. This kit uses an analogue of the nucleotide thymidine called bromodeoxyuridine (BrdU). This nucleotide analogue is taken up by proliferating cells and incorporated into their DNA. The cells that have incorporated BrdU can then be detected using anti-BrdU antibodies and flow cytometry. BrdU labelling and detection was performed as per the manufacturer's protocol by Dr B. Zhu (Zhu *et al.*, 2008). Briefly, mice were supplied with 0.8 mg/ml BrdU in drinking water for 9 days and then

splenocytes were extracted. The splenocytes were first stained with APC-conjugated anti-CD44 antibody and either PE-conjugated anti-CD4 or anti-CD8 antibody, and then fixed, permeabilised and treated with DNAse using the buffers supplied in the BrdU flow kit (BD Biosciences). The cells were then stained with FITC-conjugated anti-BrdU antibody and the percentage of T cells that had incorporated BrdU was analysed by flow cytometry.

Examination of the proliferation of adoptively transferred $CD4^+$ or $CD8^+$ T cells in wild type C57BL/6 mice was performed by Dr S. Li at Brunel University using Carboxyfluorescein succinimidyl ester (CFSE) labelling (Zhu *et al.*, 2008). To label the cells with CFSE, they were first treated with Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE); a cell membrane permeable fluorescent molecule. Upon entering the cell, esterases cleave the acetate groups from CFDA-SE generating CFSE which is cell membrane impermeable; hence cells retain CFSE within their cytoplasm. Upon cyokinesis, the CFSE molecules are divided amongst the two daughter cells leading to a two fold decrease in the relative fluorescence intensity compared to the parent cell. Thus, the number of cell divisions that the cells have undergone, and hence their proliferation, can be determined by analysing CFSE dilution. $CD4^+$ or $CD8^+$ T cells were extracted from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice, labelled with CFSE and transferred into 12-week-old C57BL/6 mice. Three to four weeks later, $CD4^+$ or $CD8^+$ T cells were purified from the spleens of recipients and stained with PE-conjugated anti-CD44 antibody and CFSE dilution was analysed.

2.16 Analysis of autoimmune disease

Histological analysis was performed by Professor J. Martin and colleagues of the Pathology department, Queen Mary, University of London (Zhu *et al.*, 2008). For

immunohistochemistry, paraffin embedded kidney sections were deparaffinised and rehydrated by incubating first in xylene and then decreasing concentrations of ethanol. Antigen retrieval was performed by boiling the slides in 10mM sodium citrate pH 6.0 for 20 minutes. The slides were then blocked with 3% Bovine Serum Albumin (BSA) in PBS and then stained with rabbit anti-human CD3 (DAKO) and rat anti-mouse B220 (BD biosciences) in 3% BSA in PBS. After washing, sections were stained with Alexa Fluor 594-conjugated donkey anti-rabbit IgG and FITC-conjugated donkey anti-rat IgG secondary antibodies (both Jackson Immunoresearch), followed by counterstaining with DAPI. Detection of immunoglobulin deposition in the glomeruli, detection of proteinuria and analysis of immunoglobulin subclass distribution and anti-nuclear antibodies was performed by Dr S. Li at Brunel University (Zhu *et al.*, 2008).

2.17 Retroviral transduction

Retroviruses infect mammalian cells and integrate their DNA into the host genome. This fact makes them a useful tool for the long term expression of exogenous proteins in the cell. Current retroviral expression systems use a system where the *gag, pol* and *env* gene products, which are required to produce mature viral particles that are capable of infecting target cells and integrating into their genome, are provided *in trans* by a packaging cell line (Pear *et al.*, 1993; Shaw *et al.*, 1997; Swift *et al.*, 2001). The expression of the *gag, pol* and *env* gene products *in trans* ensures that once the first target cell has been infected no infectious viral particles are produced. The gene of interest is cloned between two viral Long Terminal Repeat (LTR) regions which serve to facilitate expression of the integrated provirus. In this study the vector pBABE-Egr-2-EGFP and the empty control vector pBABE-EGFP (Morgenstern and Land, 1990) (both kindly provided by Professor K. Jessen, University College London) were used.

These constructs were transfected into the packaging cell line Phoenix-E (Shaw *et al.*, 1997; Swift *et al.*, 2001) (also a kind gift from Professor K. Jessen, University College London) using FuGENE (Roche) according to the manufacturer's instructions. FuGENE is a lipid based reagent that coats the DNA allowing it to pass through the plasma membrane and into the cell. Once the constructs are inside the cell, viral RNA is expressed from the vector. A packaging sequence present in the viral RNA, termed ψ, is recognised by *gag* gene products which package the viral RNA with *gag*, *pol* and *env* gene products into infectious virions which then bud from the cell. 48 hours after transfection the supernatant, containing retroviral particles, was harvested from the cells. This supernatant was then used to infect the target cells using Retronectin (Takara) according to the manufacturer's instructions. Retronectin is a recombinant fragment of fibronectin that can be bound by cell surface receptors and by retroviral particles. Thus this molecule serves to bring the virus into contact with the cell. 24 well plates were coated with 25μg of Retronectin for 2 hours at room temperature. After washing, retroviral supernatant was added to the wells and incubated at 32°C for 4 hours. During this time the viral particles bind to the Retronectin protein. The plates were then washed with PBS and $7.5x10^4$ MF2.2D9 T hybridoma cells added. Once the virus has infected the cell the viral RNA is reverse transcribed and the resulting cDNA integrated into the host genome. The viral LTRs then direct the transcription of the gene of interest. The pBABE-Egr-2-EGFP and pBABE-EGFP vectors also encode EGFP; EGFP is derived from a protein from Jellyfish that fluoresces when excited by ultraviolet (UV) light. Thus to detect whether transduction has occurred EGFP expression can be monitored. Cells were expanded and EGFP positive transduced cells were isolated by FACS (see section 2.14).

2.18 Electrophoretic Mobility Shift Assay

Electrophoretic Mobility Shift Assay (EMSA) is a technique that detects protein binding to a DNA sequence. The DNA oligonucleotide is labelled with a radioisotope and then incubated with a protein mixture. The samples are then run on a non-denaturing Polyacrylamide gel. If the DNA oligonucleotide is bound by a protein its migration through the gel will be retarded; hence this technique allows the investigation of Protein DNA interactions. Oligonucleotides matching the sense and antisense strands of the sequence of interest from the p21 promoter (p21 probe (5'-GGGCTGCCTCTGAGGGGGCGGGGC-3')) were designed with an extra 3 G nucleotides at the 5' ends. 40μM of these sense and antisense oligonucleotides were mixed together, heated to 95°C for 5 minutes and then allowed to cool to room temperature. Heating to 95°C denatures any secondary structures that may be present in the oligonucleotides so that they can bind to the complementary sequence upon cooling. This process results in a double stranded probe that has 3 G nucleotide overhangs at both 5' ends. These overhangs were then filled in using the Ready-to-Go DNA labelling beads (Amersham) according to the manufacturer's instructions. Radioactive [α-^{32}P]dCTP nucleotides were added to the mixture and the Klenow fragment from DNA polymerase I present in the kit fills in the overhanging ends of the probes with this nucleotide, resulting in the radioactive labelling of the probe. Labelled probes were then purified using ProbeQuant G-50 microcolumns (Amersham) according to the manufacturer's instructions. These gel filtration columns contain Sephadex G-50 beads. Single nucleotides can enter the pores of these beads but the larger probe cannot. Therefore, the probe passes through the column faster than the nucleotides and is eluted first, while the single nucleotides are retained in the column. After purification, the labelled probe was incubated with nuclear extracts from stimulated and unstimulated

MF2.2D9 cells in a buffer containing 10mM HEPES pH 7.5, 50mM KCl, 2.5mM $MgCl_2$, 10mM DTT, 1µg poly(dI-dC), 10% glycerol, 0.5mM $ZnCl_2$ and 5mM spermidine for 40 minutes at room temperature. For supershift reactions anti-Egr-2 (Covance) or anti-Egr-3 (Santa Cruz Biotechnology) antibody were added after 10 minutes of incubation. The samples were electrophoresed on 5% polyacrylamide gels in 0.5x Tris Borate EDTA (TBE). The gels were dried under vacuum and exposed to autoradiographic film at −80°C overnight. The film was then developed.

2.19 Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) is a technique that enables detection of Protein-DNA interactions *in vivo*. This technique uses an antibody to specifically precipitate the protein of interest and the associated DNA. ChIP was performed according to the protocol supplied by Upstate Biotechnology. Briefly, 10^8 Egr-2 and EGFP transduced MF2.2D9 cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature. This chemical binds the chromatin together preventing the dissociation that normally occurs upon cellular lysis. Crosslinking was quenched by the addition of 125 mM glycine and the cells were then washed twice with PBS. The cells were then lysed with a buffer containing 1% SDS, 10mM EDTA, 50mM Tris pH8 and transferred to 15ml tubes. The lysates were then sonicated with a Diagenode Bioruptor to shear the DNA protein complexes into lengths of less than 1Kb. Sonication was performed for a total of 10 minutes on the High output setting with 30 second bursts of sonication separated by 30 second intervals. Sonication was verified by taking a small sample of the chromatin, reversing crosslinks by heating to 65°C overnight and running on a 1% agarose gel. 500µg of sonicated chromatin was precleared for 1 hour at 4°C with salmon sperm DNA (ssDNA)/BSA-blocked Protein A beads (Amersham). This step aims to

remove any proteins or DNA that binds non-specifically to Protein A beads. The chromatin was then used as a template for immunoprecipitation. An anti-Egr-2 or irrelevant control (anti-tapasin) antibody was added to the sonicated lysate and incubated at 4°C overnight. The anti-Egr-2 antibody will bind to the Egr-2 protein and associated DNA and this antibody-protein-DNA complex can then be precipitated by the addition of ssDNA/BSA-blocked Protein A beads. These Protein A beads consist of Protein A from *Staphylococcus Aureus* conjugated to agarose beads. Protein A binds to the Fc region of Immunoglobulin and the insoluble agarose causes the immunocomplexes to be found in the pellet after centrifugation. After the immunocomplexes were pelleted and the supernatant, which contains non-specific chromatin, was removed, the pellet was washed in buffers of increasing ionic strength. After the final wash the pellet was resuspended in elution buffer containing SDS. This SDS will denature the antibody and Protein A resulting in the release of the immunoprecipitated chromatin complexes. The crosslinks were then reversed by heating to 65°C overnight. The DNA was then purified by phenol chloroform extraction and ethanol precipitation as per the protocol in (Sambrook *et al.*, 1989) and used as template for PCR. ChIP primer sequences are shown in Table 2.3.

Primer		Sequence
p21cip1	sense	5'-ATCGGTGAAGGAGTGGGTTGGTCC-3'
	antisense	5'-ACACCTCTCGGCTGCTGCAGTTGG-3'
SOCS3	sense	5'-TGTGTACTCAAGCTGGTGCAC-3'
	antisense	5'-CATACTGATCCAGGAACTCC-3'

Table 2.3: ChIP primer sequences.

2.20 Microarray

$CD4^+$ T cells from 3 month old Egr-$2^{loxP/loxP}$ and Egr-2 cKO mice were isolated by MACS and stimulated for 6 hours with plate bound anti-CD3 or left unstimulated. Total RNA was first isolated using the trizol reagent as described above and then further purified using the Qiagen RNAeasy kit RNA cleanup protocol according to the manufacturer's instructions. The experiment was performed ten times and the trizol lysates for each condition were pooled. The purified RNA was then given to the Genome Centre, Queen Mary, University of London who processed the samples and hybridised them to Illumina MouseRef-8 BeadChip expression arrays. These arrays consist of 50-mer oligonucleotide probes directed against ~24,000 well annotated RefSeq transcripts. These arrays were manufactured by chemically synthesising the oligonucleotide probes covalently attached to 3 micron beads which are then fixed in place on the array. Upon introduction of labelled test RNA sequences, the labelled RNA will hybridise to complementary sequences present on the array and the unbound RNA is then washed away. The array is then scanned to detect the bound RNA via the fluorescent label providing a genome wide transcriptional profile for that sample. The data was first normalised using the cubic spline method and then returned to us for further analysis. We initially focussed on genes that showed a difference of at least three-fold between Egr-$2^{loxP/loxP}$ and Egr-2 cKO $CD4^+$ T cells and excluded genes that had a detection p-value greater than 0.05. The differentially expressed genes were classified into different groups depending upon their function as determined by searching the NCBI Entrez Nucleotide database. Real Time RT-PCR was performed to validate the results using the primers listed above (section 2.9.1). Based upon the results (see section 3.12) we subsequently refined our analysis and excluded genes that had a detection p-value greater than 0.01.

Chapter 3: RESULTS

3.1 Egr-2 is expressed in tolerant T cells

Microarray analysis found that Egr-2 was expressed in both naïve and tolerant Tg4 T cells 2 hours after *in vivo* antigen challenge (Anderson *et al.*, 2006). To confirm this, we performed Real Time PCR and Western blotting analysis of naïve (A) and tolerant (T) Tg4 T cells that were either unstimulated (A0 and T0) or had been treated with peptide *in vivo* 2 hours previously (A2 and T2). Tolerance induction was confirmed by the cytokine mRNA profile; low IL-2 and high IL-10 for T2 compared to high IL-2 and low IL-10 for A2 (data not shown). We found that Egr-2 was indeed expressed at both the mRNA and protein levels 2 hours after *in vivo* antigen challenge in both naïve and tolerant T cells (Figure 3.1). Thus, unlike many other transcription factors which are not activated (Anderson *et al.*, 2006), Egr-2 is expressed in tolerant T cells following TCR engagement suggesting it may be involved in the maintenance of tolerance.

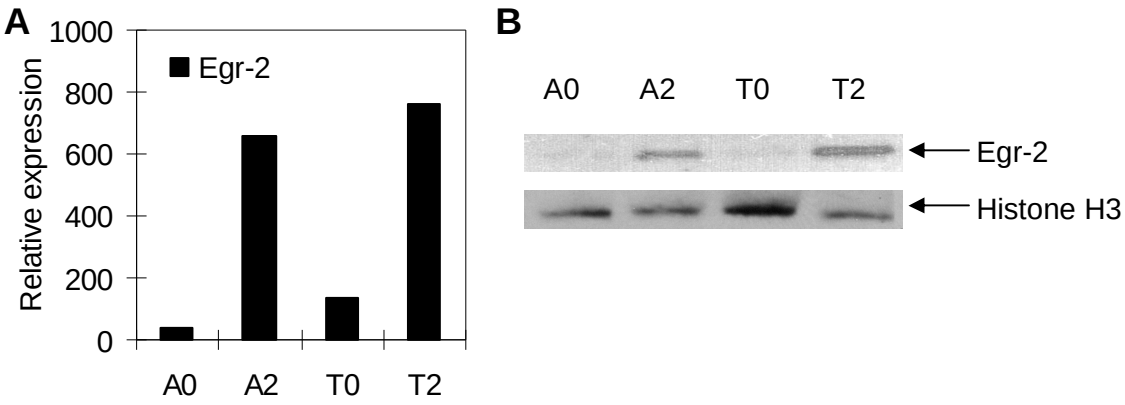

Figure 3.1: Egr-2 expression in tolerant T cells. Transgenic Tg4 T cells were tolerised by 10 i.n. administrations of 100µg of the Ac1-9[4Y] peptide from myelin basic protein as described (Anderson *et al.*, 2006). Resting (A0 and T0) and *in vivo* stimulated (A2 and T2) naïve (A) and tolerant (T) $CD4^+$ Tg4 T cells were isolated and Egr-2 expression was examined by Real Time PCR (**A**) and western blot (**B**). Histone H3 serves as a loading control.

3.2 Generation of Egr-2 cKO and Tg mice

Egr-2 KO mice die soon after birth due to defects in brain development (Schneider-Maunoury *et al.*, 1993) precluding analysis of T cells in these mice. Therefore, to investigate the roles of Egr-2 in T cells *in vivo*, we generated mice in which the Egr-2 gene was deleted only in $CD2^+$ lymphocytes (see methods). Egr-$2^{loxP/loxP}$ mice were crossed with hCD2-Cre mice and Egr-$2^{loxP/loxP}$ hCD2-Cre mice were identified by PCR (Figure 3.2, A). Egr-$2^{loxP/loxP}$ hCD2-Cre mice were generated at the expected frequencies and displayed no developmental defects. To confirm that T cells from Egr-$2^{loxP/loxP}$ hCD2-Cre mice have deleted Egr-2, splenic $CD4^+$ T cells were isolated and stimulated with anti-CD3 and anti-CD28 and Egr-2 expression was examined by Real Time PCR and western blot (Figure 3.2, B and C). Figure 3.2 shows that T cells from Egr-$2^{loxP/loxP}$ mice strongly induce Egr-2 expression after stimulation while T cells from Egr-$2^{loxP/loxP}$ hCD2-Cre mice have no detectable Egr-2 protein expression confirming that Cre mediated deletion has occurred effectively. However, there was very weak mRNA expression, which may be due to incomplete Cre mediated deletion in a small proportion of cells. Nevertheless, the Egr-$2^{loxP/loxP}$ hCD2-Cre mice are effectively a conditional T cell KO and are hereafter designated Egr-2 cKO.

Using a GFP reporter gene Kioussis and colleagues demonstrated that the hCD2 construct also directs expression of the transgene in mature B cells, albeit at lower levels than in mature T cells (de Boer *et al.*, 2003). Therefore we examined the expression of Egr-2 in B cells from Egr-2 cKO mice. B cells were extracted from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice and stimulated with an anti-surface Ig antibody. In contrast to the results from T cells, substantial levels of Egr-2 transcripts could still be detected in B cells from Egr-2 cKO mice after stimulation (Figure 3.2, D). These results indicate

that deletion of Egr-2 is inefficient in B cells in Egr-2 cKO mice perhaps due to the weak activity of the hCD2 promoter in mature B cells (de Boer *et al.*, 2003).

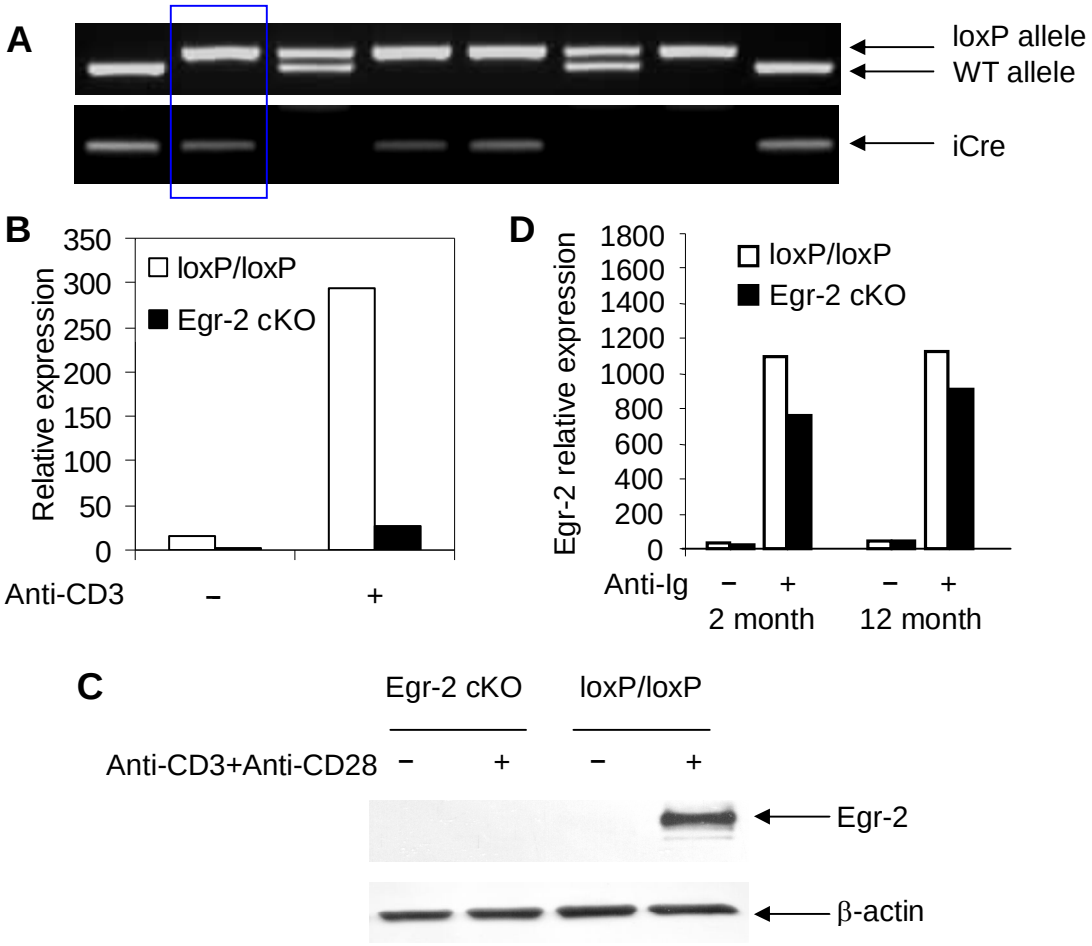

Figure 3.2: Generation of Egr-2 cKO mice. Egr-2$^{loxP/loxP}$ mice were crossed with hCD2-Cre mice to generate Egr-2$^{loxP/loxP}$ hCD2-Cre (Egr-2 cKO) mice. (**A**) Genomic DNA was extracted from tail samples and Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice were identified by PCR. The box highlights an Egr-2 cKO mouse. [(**B**) and (**C**)] Splenic CD4^{+} cells were isolated from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice and stimulated with anti-CD3 and anti-CD28 for 6 (**B**) or 16 hours (**C**). Egr-2 expression was examined by Real Time PCR (**B**) and western blot (**C**). β-actin serves as a loading control. (**D**) Splenic B220^{+} cells were isolated from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice of different ages as indicated. Cells were then stimulated with anti-surface Ig antibody for 6 hours and Egr-2 expression examined by Real Time PCR.

To complement these Egr-2 cKO mice we decided to generate transgenic mice which constitutively over-express Egr-2 in CD2^{+} lymphocytes. The hCD2 construct that drives

Cre expression in Egr-2 cKO mice was used to drive Egr-2 expression in these transgenic mice so that the populations that have deleted Egr-2 in Egr-2 cKO mice will over-express Egr-2 in these transgenic mice. These Egr-2 transgenic (Tg) mice were generated by standard transgenic techniques (see methods). The progeny were checked for the presence of the hCD2 Egr-2 construct by PCR and two mice, Tg14 and Tg18, were found to have integrated the targeting construct into their genome. Egr-2 Tg mice were generated from these founders at the expected frequencies and displayed no obvious gross abnormalities. Egr-2 Tg mice were identified by PCR (Figure 3.3, A). To confirm that thymocytes from these Egr-2 Tg mice constitutively express Egr-2, total thymocytes were extracted from Egr-2 Tg and wild type mice and Egr-2 expression was examined by Real Time PCR and western blot (Figure 3.3, B and C). Figure 3.3 shows that, while thymocytes from wild type mice express low levels of Egr-2, thymocytes from Egr-2 Tg mice have greatly increased expression. The Egr-2 Tg14 line expressed greater levels of Egr-2 than the Tg18 line (Figure 3.3, B). Therefore, Egr-2 Tg mice constitutively express Egr-2 in thymocytes indicating that the hCD2 construct directs Egr-2 expression in cells of the T cell lineage.

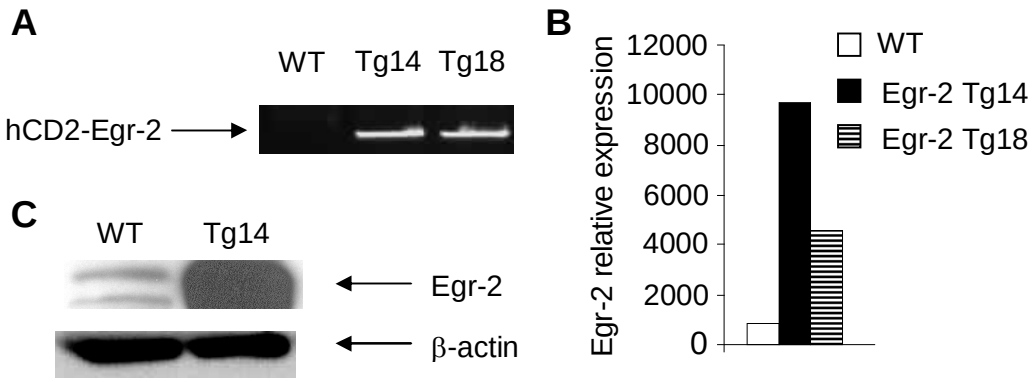

Figure 3.3: Generation of Egr-2 Tg mice. Egr-2 Tg mice were generated as described in the methods section. (**A**) Genomic DNA was extracted from tail samples and hCD2-Egr-2^{+} (Egr-2 Tg) mice were identified by PCR. [(**B**) and (**C**)] Thymocytes were isolated from Egr-2 Tg and wild type mice and Egr-2 expression was examined by Real Time PCR (**B**) and western blot (**C**). β-actin serves as a loading control.

3.3 Egr-2 in T cell development

Next we examined T cell development in Egr-2 cKO mice. Egr-2 has been implicated in β-selection and the related protein Egr-1 is involved in positive selection (Miyazaki and Lemonnier, 1998; Bettini *et al.*, 2002; Carleton *et al.*, 2002; Carter *et al.*, 2007). However, we did not detect any significant changes in the percentages or absolute numbers of the major thymocyte subsets in Egr-2 cKO mice; DN, DP and SP cells were present at frequencies similar to Egr-$2^{loxP/loxP}$ mice (Figure 3.4, A and B), nor were there any changes in DN cells consistent with a defect in β-selection (Figure 3.4, C and D). In addition we found that $CD4^{+}CD25^{+}FoxP3^{+}$ T_{Reg} cells were generated in Egr-2 cKO mice thymi at frequencies similar to Egr-$2^{loxP/loxP}$ mice (Figure 3.4, E). Therefore, T cell development appears to be normal in Egr-2 cKO mice.

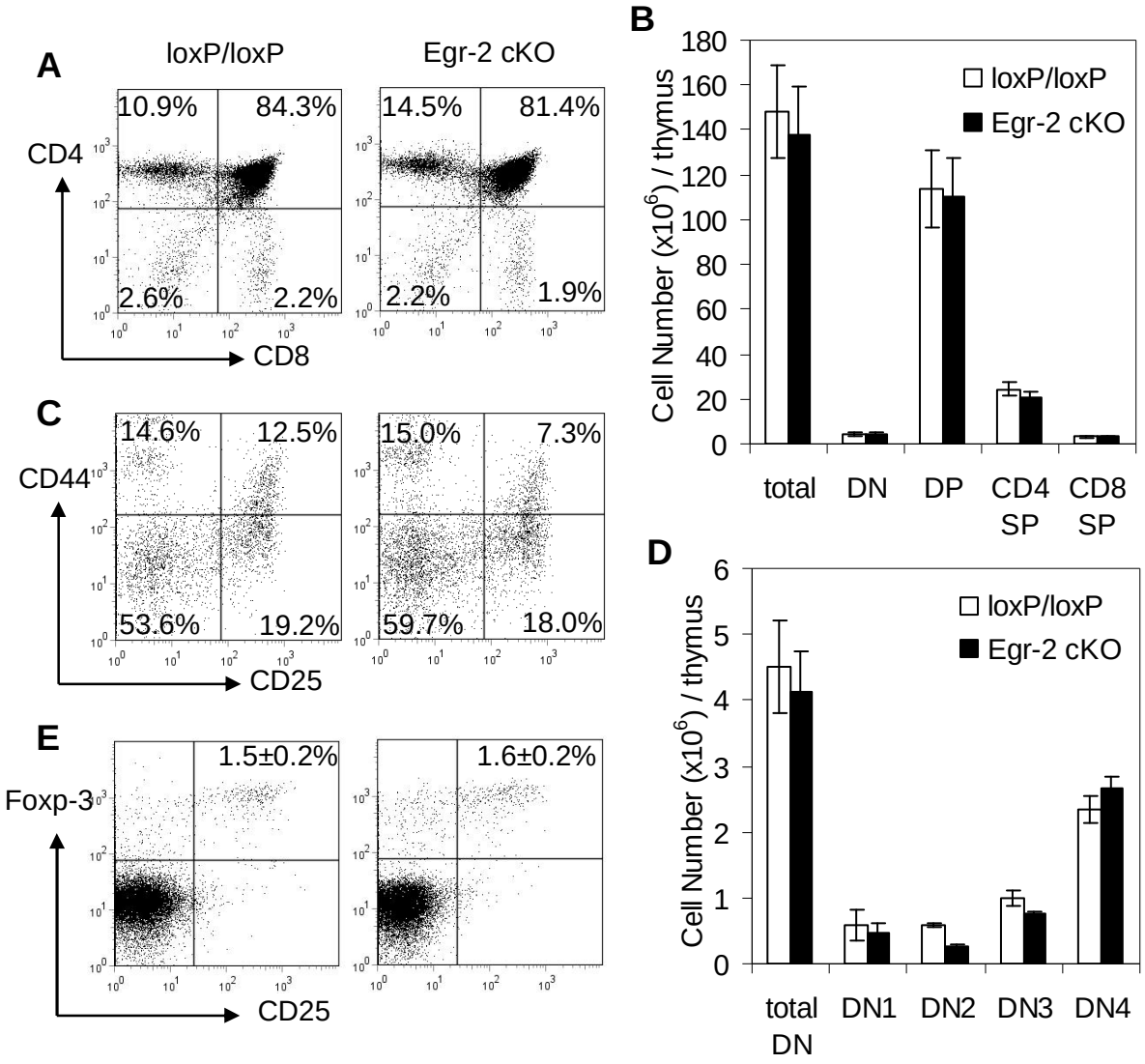

Figure 3.4: T cell development in Egr-2 cKO mice. Thymocytes were extracted from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice, stained with the indicated fluorochrome labelled antibodies and analysed by flow cytometry. [(**A**) and (**B**)] Percentages (**A**) and absolute numbers (**B**) of the major thymocyte subsets in Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice. [(**C**) and (**D**)] Percentages (**C**) and absolute numbers (**D**) of the DN subsets in Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice. In (**A**) and (**C**) the data is representative of at least 3 mice of each genotype at three to four months of age. In (**B**) and (**D**) the data is the mean ± standard deviation from 3 mice of each genotype at three to four months of age. (**E**) Examination of CD4$^+$ T_{Reg} cells. Expression of CD25 and FoxP3 was examined on gated CD4$^+$ cells. The numbers indicate the percentage of cells positive for both FoxP3 and CD25.

In contrast to the apparently normal thymocyte development in Egr-2 cKO mice, Egr-2 Tg mice had striking alterations in thymocyte development. The thymus was much smaller in both Egr-2 Tg mice lines; in Tg18 it was around half the size of wild type while Tg14 was even smaller, around one third to one quarter of the size of that in wild type (Figure 3.5, A). Consistent with this reduced thymic size, the total number of thymocytes in Egr-2 Tg14 mice was also reduced to approximately one third of wild type mice (Figure 3.5, C). Although there was some difference between the two transgenic lines in terms of the exact percentages of the major thymocyte subsets, both had a similar pattern (see Figure 3.5, B). In both transgenic lines the percentage of DP cells was decreased; with ~50% in Tg18 and ~20% in Tg14 compared to ~80% in wild type mice. In addition, the percentages of DN and, in particular, $CD8^+$ SP cells were dramatically increased. In wild type mice the DN population accounted for only ~2.5% of thymocytes but in the Egr-2 Tg DN cells accounted for 10-15% of thymocytes. Even more striking was the increase in $CD8^+$ SP cells; this population accounted for 2-4% of wild type but in Egr-2 Tg18 it was 15-20% while in Egr-2 Tg14 mice $CD8^+$ SP cells were the predominant population accounting for 40-50% of total thymocytes. While the absolute number of DN cells in Egr-2 Tg14 mice was similar to wild type, the $CD4^+$ SP and DP cells were reduced in Egr-2 Tg14 mice consistent with the reduction in total thymocyte numbers. In stark contrast the absolute number of $CD8^+$ SP cells was increased four-fold in Egr-2 Tg14 mice compared to wild type despite the decrease in total thymocyte numbers (Figure 3.5, C).

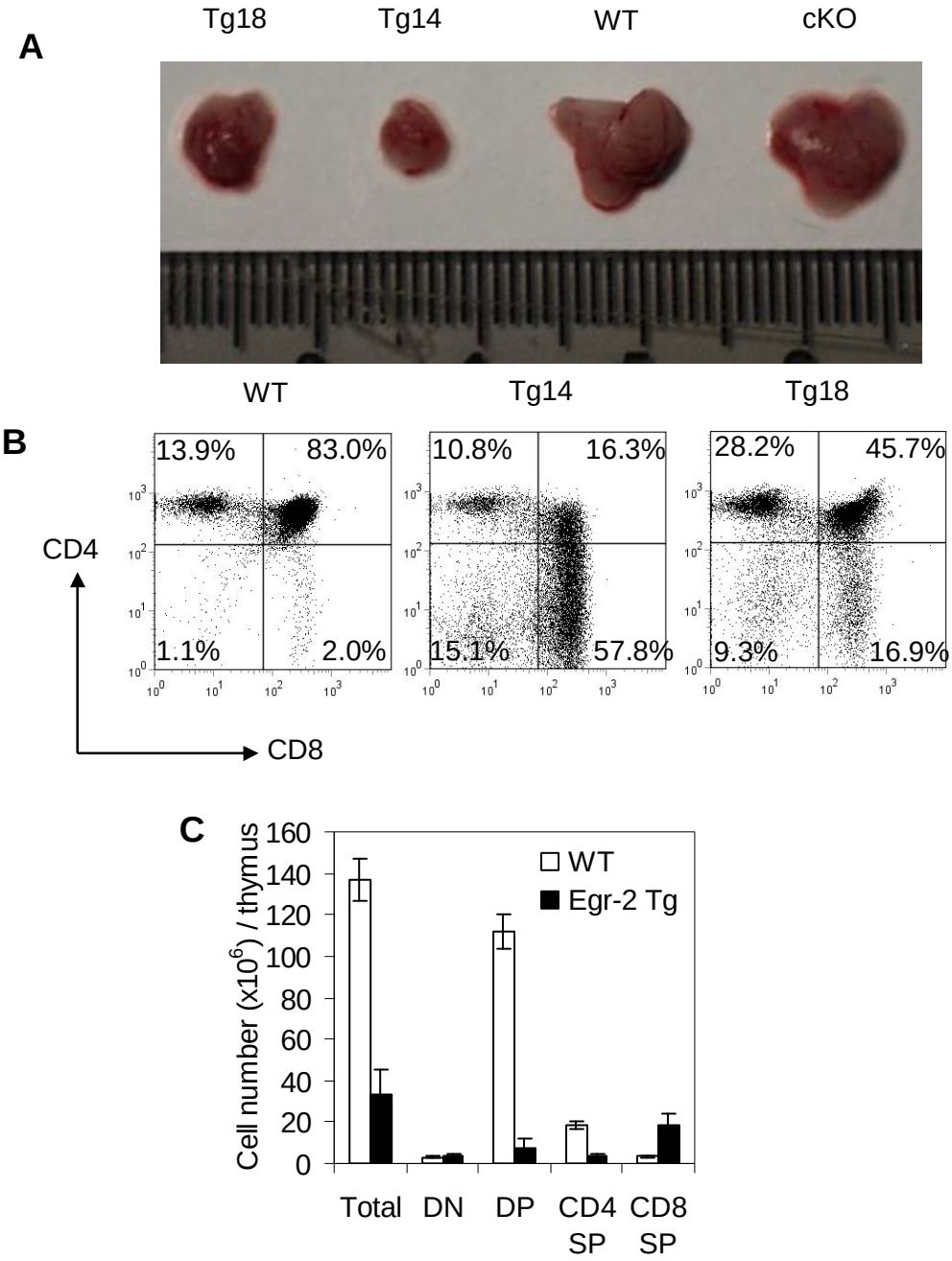

Figure 3.5: T cell development in Egr-2 Tg mice. (**A**) Thymus size in Egr-2 Tg14 and Tg18 compared to wild type and Egr-2 cKO mice. [(**B**) and (**C**)] Thymocytes were extracted from Egr-2 Tg and wild type mice, stained with the indicated fluorochrome labelled antibodies and analysed by flow cytometry. (**B**) Percentages of the major thymocyte subsets in Egr-2 Tg14, Tg18 and wild type mice; the data is representative of at least 2 mice of each genotype at three to four months of age. (**C**) Absolute numbers of the major thymocyte subsets in Egr-2 Tg14 and wild type mice; the data is the mean ± standard deviation from 3 mice of each genotype at three to four months of age.

To further investigate the expanded $CD8^+$ SP population in Egr-2 Tg mice, the expression of the mature T cell markers CD3, TCR-β and CD5 was examined. While more than 80% of wild type $CD8^+$ SP cells expressed CD3, TCR-β or CD5, only 20-50% of the $CD8^+$ SP population from Egr-2 Tg14 mice expressed these markers (Figure 3.6, C). In contrast, the expression of these markers by $CD4^+$ SP cells was similar in wild type and Egr-2 Tg14 mice with around 90% of $CD4^+$ SP cells expressing CD3, TCR-β or CD5 (Figure 3.6, C). Thus, although the absolute number of $CD8^+$ cells in Egr-2 Tg14 mice was increased, the absolute number of mature $CD8^+$ SP cells was actually decreased in Egr-2 Tg14 mice compared to wild type (~$3x10^6$ in Egr-2 Tg14 compared to ~$5x10^6$ in wild type). Therefore, the expansion of the $CD8^+$ SP population in Egr-2 Tg mice is due to increased numbers of $CD8^+$ ISP rather than mature $CD8^+$ SP cells.

While the total number of DN cells was within normal parameters, a detailed examination of the DN subsets revealed an increase in both the percentage and absolute number of DN4 cells (Figure 3.6, A and B).

Therefore the main defects in Egr-2 Tg mice appear to be accumulation of cells in the DN4 and $CD8^+$ ISP compartments, those stages immediately following passage through the β-selection checkpoint. These results are markedly similar to those seen in Egr-1 Tg and Egr-3 Tg mice (Miyazaki, 1997; Xi and Kersh, 2004) supporting the idea that Egr proteins have overlapping functions during T cell development (Carter *et al.*, 2007).

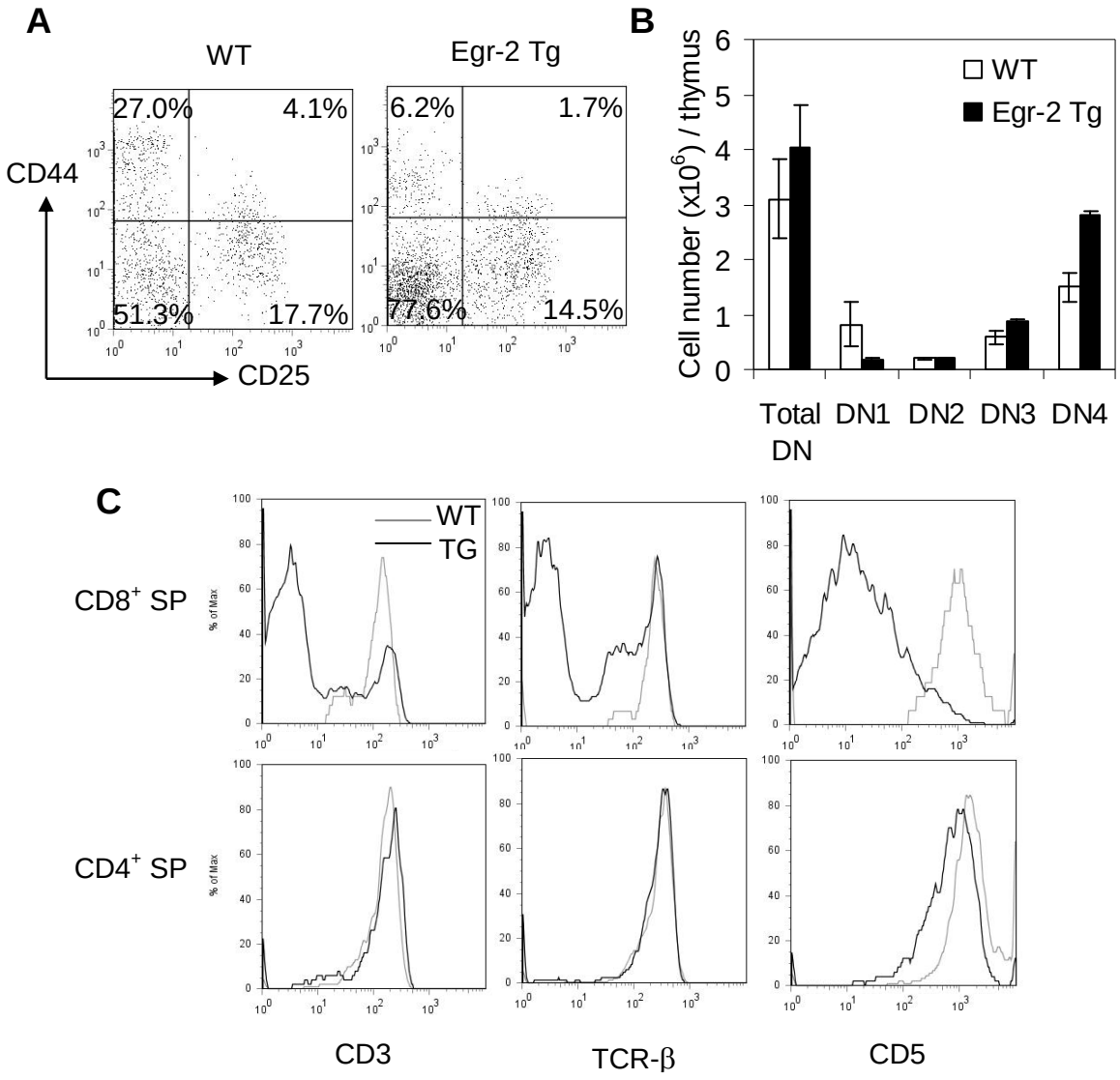

Figure 3.6: Thymic CD8$^+$ and DN populations in Egr-2 Tg mice. Thymocytes were extracted from Egr-2 Tg14 and wild type mice, stained with the indicated fluorochrome labelled antibodies and analysed by flow cytometry. [(**A**) and (**B**)] Percentages (**A**) and absolute numbers (**B**) of the DN subsets in Egr-2 Tg14 and wild type mice. In (**A**) the data is representative of 3 mice of each genotype at three to four months of age. In (**B**) the data is the mean ± standard deviation from 3 mice of each genotype at three to four months of age. (**C**) Expression of CD3, TCR-β and CD5 was examined on gated CD8$^+$ SP (upper) or CD4$^+$ SP (lower) thymocytes from Egr-2 Tg14 and wild type mice; the data is representative of 2 mice of each genotype at three to four months of age.

3.4 Peripheral T cell populations

Although T cell development appeared normal in Egr-2 cKO mice, the total number of $CD3^+$ T cells in the spleen was decreased; the total number of cells in three to four month old Egr-2 cKO mice was only two-thirds of that seen in age matched Egr-$2^{loxP/loxP}$ mice (Figure 3.7, A and C). The numbers of $CD4^+$ and $CD8^+$ cells in these young Egr-2 cKO mice were also decreased compared to Egr-$2^{loxP/loxP}$ mice (Figure 3.7, B and C); although this appeared to affect $CD8^+$ cells more than $CD4^+$ cells. Next we examined regulatory T cells in Egr-2 cKO mice. We found that $CD4^+CD25^+FoxP3^+$ T_{Reg} cells were present in the periphery in Egr-2 cKO mice at similar frequencies to Egr-$2^{loxP/loxP}$ mice (Figure 3.7, D). We then examined the expression of activation markers by $CD4^+$ cells. The percentage of $CD4^+$ cells expressing the activation markers CD25 and CD69 was normal in young Egr-2 cKO mice (Figure 3.7, E). However, there were slight increases in the percentage of cells expressing high levels of the marker CD44 and in the percentage of cells expressing low levels of the marker CD62L (Figure 3.7, E).

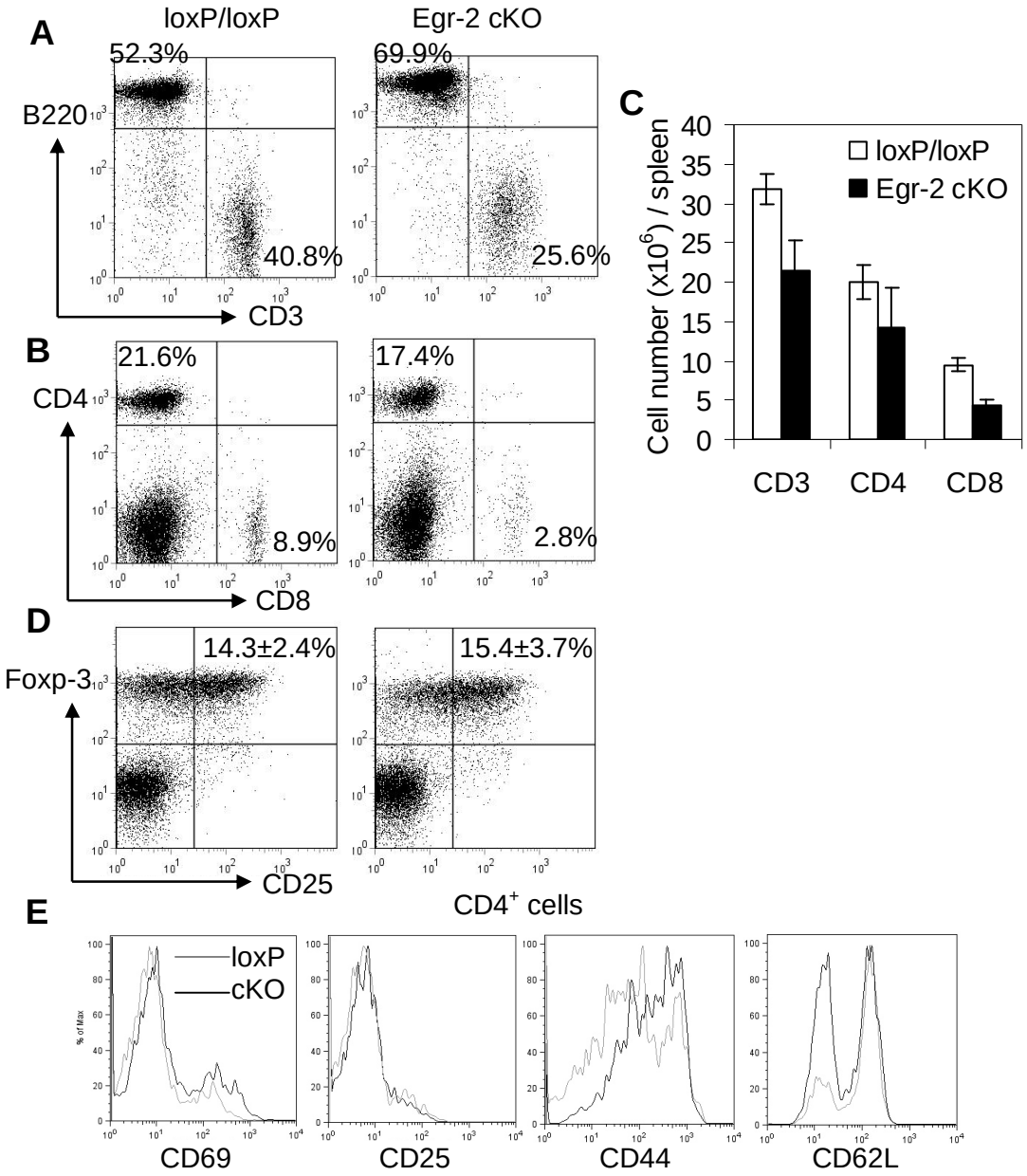

Figure 3.7: Splenic B cell and T cell populations in Egr-2 cKO mice. Splenocytes were extracted from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice, stained with the indicated fluorochrome labelled antibodies and analysed by flow cytometry. [(**A**) and (**B**)] Representative flow cytometry analysis of B220$^+$ and CD3$^+$ cells (**A**) and CD4$^+$ and CD8$^+$ cells (**B**). (**C**) Absolute numbers of CD3$^+$ cells, CD4$^+$ cells and CD8$^+$ cells in Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice. In (**C**) the data is the mean ± standard deviation from 5 mice of each genotype at three to four months of age. (**D**) Examination of CD4$^+$ T_{Reg} cells. Expression of CD25 and FoxP3 was examined on gated CD4$^+$ cells. The numbers indicate the percentage of cells positive for both FoxP3 and CD25. (**E**) Expression of CD69, CD25, CD44 and CD62L was examined on gated CD4$^+$ cells. The data in (**A**), (**B**), (**D**) and (**E**) is representative of at least 3 mice of each genotype.

Despite the alterations in thymocyte development, the size of the spleen and the total number of splenocytes in Egr-2 Tg14 mice were similar to wild type (data not shown). However, there were marked changes in the composition of cells in Egr-2 Tg mice. While in wild type mice the majority of splenocytes, more than 90%, were either $B220^+$ or $CD3^+$, in Egr-2 Tg mice there were a considerable number of $CD3^-B220^-$ cells; around 20-30% (Figure 3.8, A). Staining of total splenocytes with CD4 and CD8 revealed that the percentage of cells that were $CD4^+$ was reduced by about a third while the percentage of $CD8^+$ cells was markedly increased in Egr-2 Tg mice with a 2.5-3 fold increase compared to wild type (Figure 3.8, B). Triple staining of splenocytes with CD3, CD4 and CD8 antibodies revealed the presence of a large population of $CD8^+CD3^-$ cells in Egr-2 Tg mice that was not present in wild type mice; this $CD8^+CD3^-$ population accounted for 50-60% of the peripheral $CD8^+$ population in Egr-2 Tg mice (Figure 3.8, C). In contrast, there were no $CD4^+CD3^-$ cells in either Egr-2 Tg or wild type mice (Figure 3.8, D). Therefore, the presence of $B220^-CD3^-$ cells and the increase in the percentage of $CD8^+$ cells in the spleen of Egr-2 Tg mice is due to the existence of these aberrant $CD8^+CD3^-$ cells.

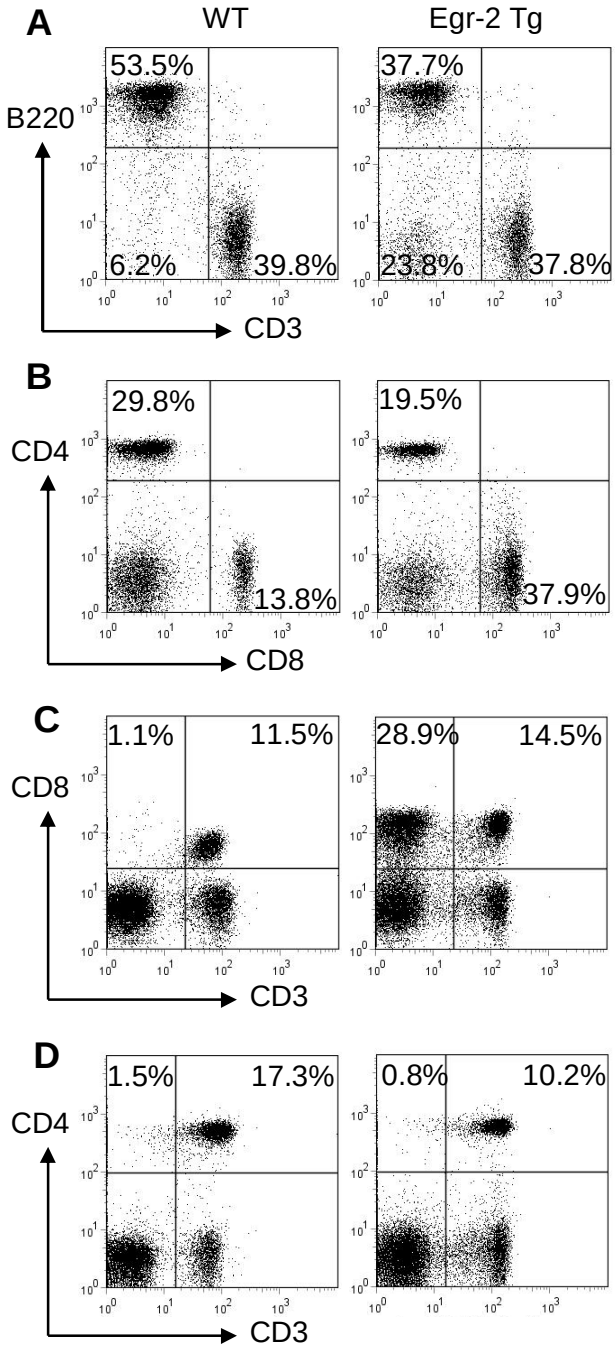

Figure 3.8: Splenic B cell and T cell populations in Egr-2 Tg mice. Splenocytes were extracted from wild type and Egr-2 Tg14 mice, stained with the indicated fluorochrome labelled antibodies and analysed by flow cytometry. [(**A**) – (**D**)] Representative flow cytometry analysis of $B220^+$ and $CD3^+$ cells (**A**), $CD4^+$ and $CD8^+$ cells (**B**), $CD3^+$ and $CD8^+$ cells (**C**) and $CD3^+$ and $CD4^+$ cells (**D**). The data is representative of at least 2 mice of each genotype.

To further investigate these aberrant $CD8^+$ cells we examined the expression of the mature T cell markers CD3, TCR-β and CD5. While more than 90% of wild type $CD8^+$ splenocytes expressed CD3, TCR-β or CD5, only around half of the $CD8^+$ population from Egr-2 Tg mice expressed CD3 or TCR-β while only ~20% were $CD5^+$ (Figure 3.9, A). In contrast, the expression of these markers by $CD4^+$ splenocytes was similar in wild type and Egr-2 Tg mice with more than 90% of $CD4^+$ cells expressing CD3, TCR-β or CD5 (Figure 3.9, A). To determine whether these $CD8^+CD3^-$ cells were of a different lineage, we investigated the expression of NK cell markers and markers typically found on cells of the innate immune system. We found that only ~2% of the $CD8^+$ cells in the spleen of Egr-2 Tg mice expressed either CD11b or NK1.1 (Figure 3.9, B). This was not due to a failure of antibody staining since there were distinct $CD8^-CD11b^+$ and $CD8^-NK1.1^+$ populations (data not shown). Similar results were seen for CD11c (data not shown). Therefore, these aberrant $CD8^+$ cells resemble $CD8^+$ ISP thymocytes rather than mature $CD8^+$ T cells or cells of the innate immune system.

In light of these gross abnormalities in T cell development and the presence of these aberrant $CD8^+CD3^-$ cells in the periphery, we felt that further comparison with the Egr-2 cKO would not be informative.

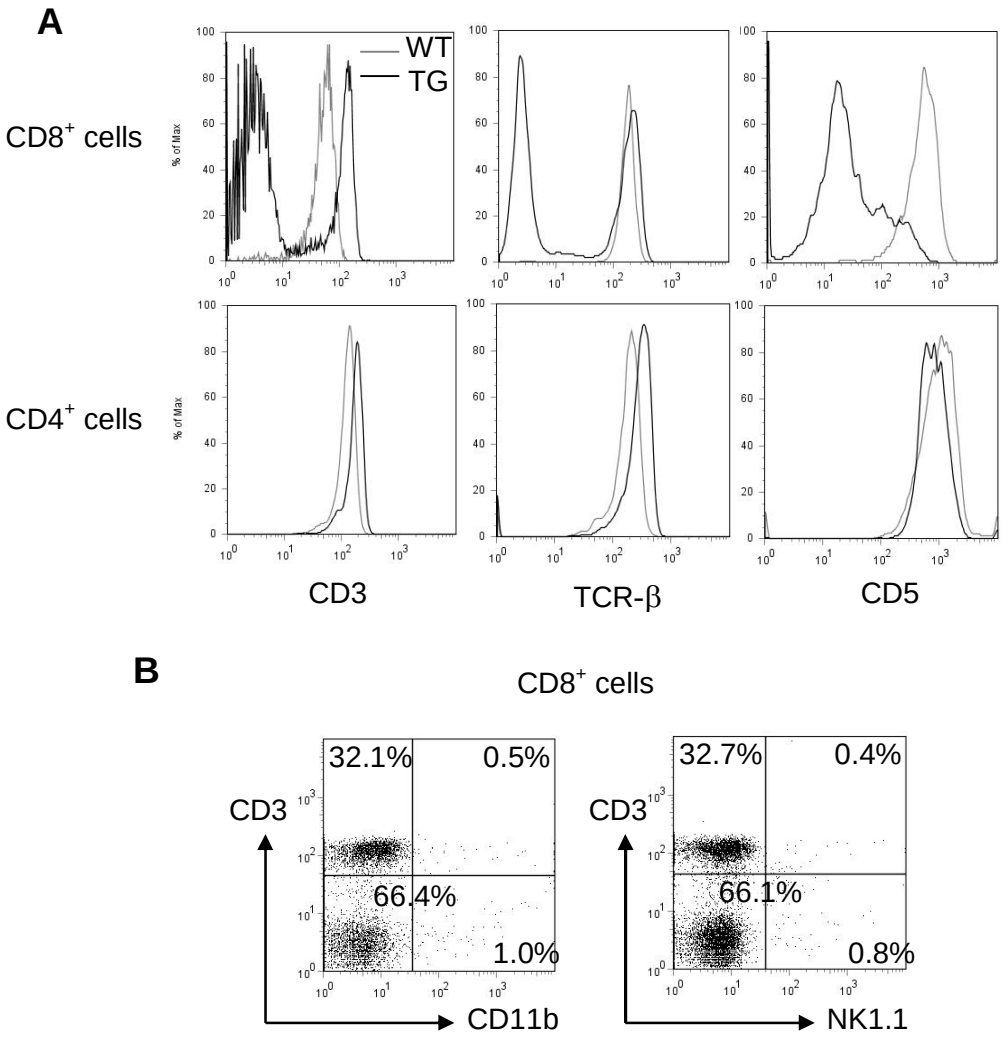

Figure 3.9: Aberrant splenic CD8$^+$ population in Egr-2 Tg mice. Splenocytes were extracted from wild type and Egr-2 Tg14 mice, stained with the indicated fluorochrome labelled antibodies and analysed by flow cytometry. (**A**) Expression of CD3, TCR-β and CD5 was examined on gated CD8$^+$ (upper) or CD4$^+$ (lower) splenocytes from Egr-2 Tg and wild type mice; the data is representative of 2 mice of each genotype at three to four months of age. (**B**) Expression of CD3, CD11b and NK1.1 was examined on gated CD8$^+$ splenocytes from Egr-2 Tg mice.

3.5 Egr-2 in T cell activation in vitro

Over-expression of Egr-2 in a T cell line resulted in decreased production of IL-2 and proliferation in response to antigen stimulation (Safford *et al.*, 2005) suggesting that Egr-2 negatively regulates TCR signalling. Therefore we investigated the response of Egr-2 cKO $CD4^+$ T cells to TCR stimulation. Egr-2 cKO and Egr-$2^{loxP/loxP}$ $CD4^+$ T cells were isolated and stimulated with different concentrations of plate-bound anti-CD3. In contrast to the previous report, we found that primary Egr-2 cKO $CD4^+$ T cells were not hyper-reactive in response to TCR stimulation *in vitro* (Figure 3.10, A). On the contrary, the Egr-2 cKO T cells were, if anything, slightly hypo-responsive, consistently exhibiting proliferative responses that were approximately 60-70% of the Egr-$2^{loxP/loxP}$ cells. Similar results were seen for CFSE labelled $CD4^+$ T cells confirming that this was not an artefact of the [^{3}H]TdR incorporation protocol (data not shown). These results suggest that Egr-2 is not simply a negative regulator of TCR signalling as has been proposed (Safford *et al.*, 2005) but has a more complex role in T cell activation. However, the different responses do not appear to be the result of altered TCR signalling as the activation of the MAPK ERK in response to TCR engagement was normal in Egr-2 cKO T cells (Figure 3.10, B). In any case, these results demonstrate that physiological levels of Egr-2 do not inhibit primary T cell activation and TCR signalling.

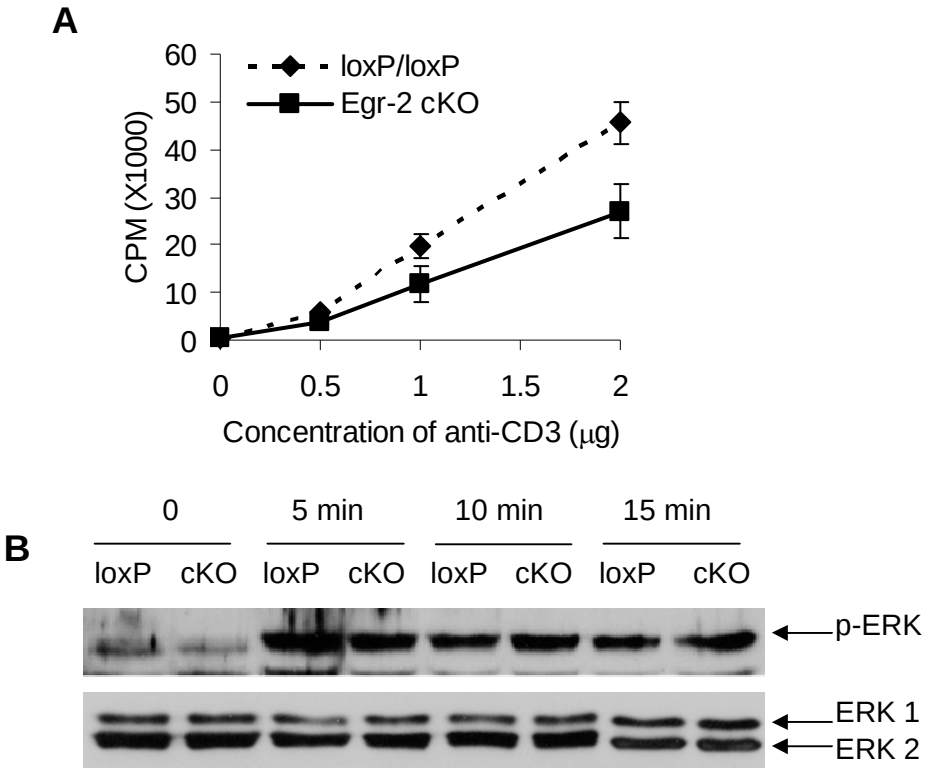

Figure 3.10: Response of Egr-2 cKO CD4$^+$ T cells to TCR stimulation. Splenic CD4$^+$ cells were isolated from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice and stimulated as indicated. (**A**) CD4$^+$ cells were stimulated with anti-CD3 at the indicated concentrations for 72 hours and proliferation analysed by [^{3}H]TdR incorporation. The data is representative of at least 3 independent experiments. (**B**) CD4$^+$ cells were stimulated with anti-CD3 for the indicated time periods and ERK activation analysed by western blot.

3.6 Apoptosis induction and FasL expression

The observed hypo-proliferation of Egr-2 cKO cells in response to TCR stimulation *in vitro* may be due to increased susceptibility to apoptosis. To investigate this possibility, CD4$^+$ cells from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice were isolated and stimulated with anti-CD3 for 48 or 72 hours and apoptosis induction was assessed by annexin V staining. We found that the percentage of annexin V positive PI negative cells was similar in Egr-2 cKO and Egr-2$^{loxP/loxP}$ T cell populations at both time points (Figure 3.11, A and B). Thus, at least under these conditions, Egr-2 cKO T cells are not more

susceptible to apoptosis. Egr-2 has been reported to regulate the expression of FasL (Mittelstadt and Ashwell, 1999). The lack of a defect in apoptosis induction argues against an obligate role for Egr-2 in regulating FasL expression after TCR stimulation. However, this may be due to the conditions used in this assay. To directly investigate the expression of FasL, $CD4^+$ cells were isolated from Egr-$2^{loxP/loxP}$ and Egr-2 cKO mice and stimulated with anti-CD3 and anti-CD28. We did not detect any difference in the expression of FasL between Egr-2 cKO and Egr-$2^{loxP/loxP}$ T cells after TCR stimulation (Figure 3.11, C). This normal expression of FasL in Egr-2 cKO T cells may be due to functional compensation by Egr-3 (Mittelstadt and Ashwell, 1998).

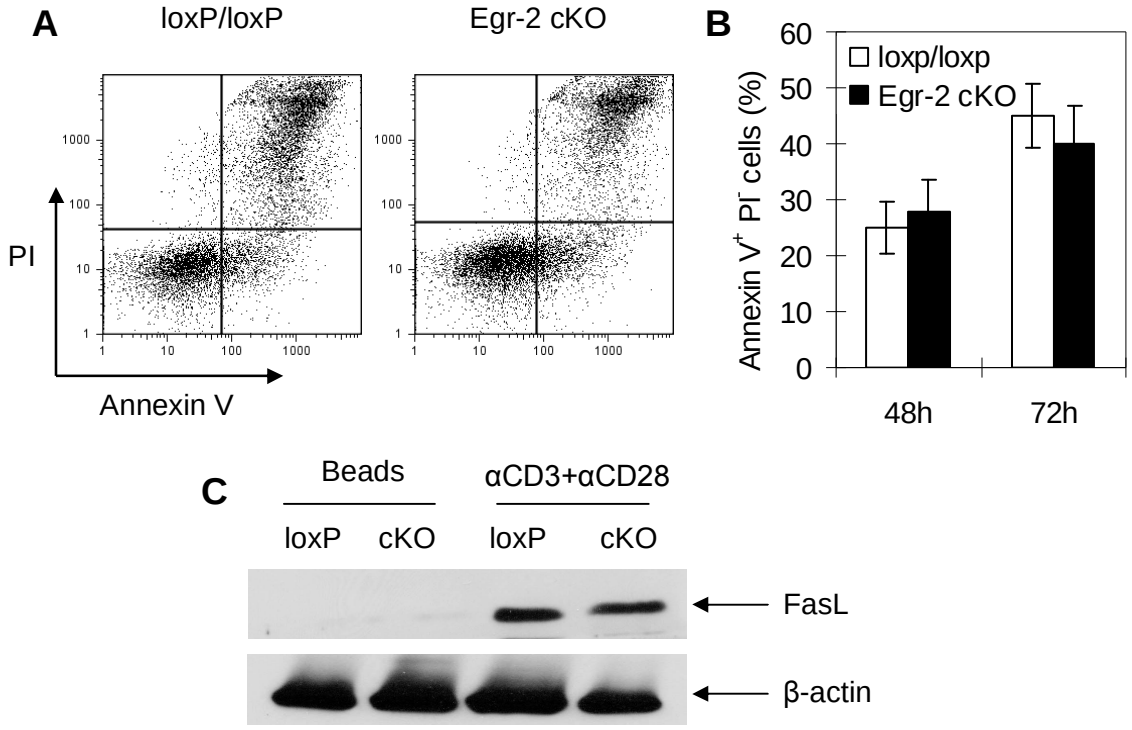

Figure 3.11: Apoptosis induction and FasL expression in Egr-2 cKO $CD4^+$ T cells. Splenic $CD4^+$ cells were isolated from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice and stimulated as indicated. [(**A**) and (**B**)] $CD4^+$ cells were stimulated with anti-CD3 for the indicated time periods and apoptosis induction was assessed by Annexin V staining. (**A**) Representative flow cytometry results after 48 hours stimulation. (**B**) The percentage of cells positive for Annexin V and negative for PI at different times after stimulation. (**C**) $CD4^+$ cells were stimulated with anti-CD3 and anti-CD28 for 16 hours and FasL expression was analysed by western blot.

3.7 Egr-2 in CD44^{high} T cells in vivo

Thus far, the results from Egr-2 cKO T cells have not shown the hyper-reactivity that we expected based upon the expression of Egr-2 in tolerant T cells and our hypothesis that Egr-2 is involved in the maintenance of tolerance (Figure 3.1). However, we did observe an increase in the percentage of $CD4^{+}CD44^{high}$ T cells in Egr-2 cKO mice. Although the percentage of $CD4^{+}CD44^{high}$ T cells was slightly increased in 3 month old Egr-2 cKO mice (Figure 3.7, E) this was even more pronounced in 8 month and 15 month old Egr-2 cKO mice (Figure 3.15, A), suggesting that Egr-2 may be involved in the homeostasis of $CD4^{+}CD44^{high}$ T cells. To investigate Egr-2 expression in $CD44^{high}$ and $CD44^{low}$ T cells, $CD4^{+}CD25^{-}CD44^{high}$ and $CD4^{+}CD25^{-}CD44^{low}$ T cells were isolated from Egr-$2^{loxP/loxP}$ mice by FACS (Figure 3.12, A). We found that the expression of Egr-2 mRNA in $CD4^{+}CD44^{high}$ T cells was around 5 times higher than that of the $CD4^{+}CD44^{low}$ population (Figure 3.12, B). Furthermore, Egr-2 protein was only detected in $CD4^{+}CD44^{high}$ T cells (Figure 3.12, C). These results demonstrate that Egr-2 is expressed in effector phenotype T cells *in vivo* in the absence of overt antigen stimulation.

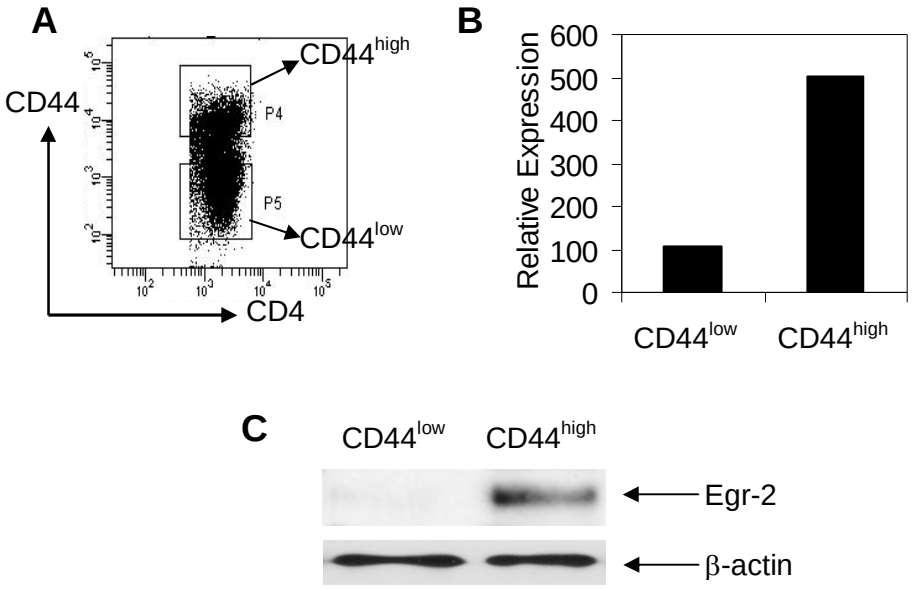

Figure 3.12: Egr-2 expression in CD44^{high} T cells *in vivo*. Splenic CD4^{+} cells were extracted from 8 month old Egr-2$^{loxP/loxP}$ mice, stained with the indicated fluorochrome labelled antibodies and CD4^{+}CD25^{-}CD44^{low} and CD4^{+}CD25^{-}CD44^{high} T cells isolated by FACS. In (**A**) the cells were first gated on CD4^{+}CD25^{-} cells. [(**B**) and (**C**)] Egr-2 expression in CD4^{+}CD25^{-}CD44^{low} and CD4^{+}CD25^{-}CD44^{high} T cells was examined by Real Time PCR (**B**) and western blot (**C**). β-actin serves as a loading control.

To investigate the effect of Egr-2 deficiency on CD44^{high} T cells, we examined their proliferation *in vivo* in the absence of experimental immunisation. Eight month old Egr-2$^{loxP/loxP}$ and Egr-2 cKO mice were supplied with BrdU for 9 days and then BrdU incorporation by splenic T cells was analysed. The proportion of BrdU-positive CD44^{low} T cells was similar in Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice and was less than 5% (Figure 3.13). However, we found that BrdU incorporation by CD44^{high} cells was increased in Egr-2 cKO mice compared to Egr-2$^{loxP/loxP}$ mice. 29.5% of CD8^{+}CD44^{high} and 21.5% of CD4^{+}CD44^{high} T cells from Egr-2 cKO mice had incorporated BrdU

(Figure 3.13). In contrast, the percentage of $CD8^+CD44^{high}$ and $CD4^+CD44^{high}$ cells that had incorporated BrdU from Egr-$2^{loxP/loxP}$ mice was 14.1% and 12.1%, respectively (Figure 3.13). One possible explanation for the hyperproliferation of $CD44^{high}$ cells is that it is due to extrinsic factors rather than a cell intrinsic defect in the control of the proliferation of $CD44^{high}$ cells. We performed adoptive transfer of isolated T cells into C57BL/6 mice in order to investigate this possibility. $CD4^+$ or $CD8^+$ T cells from seven month old Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice were labelled with CFSE and then transferred. Consistent with the increased proliferation of $CD44^{high}$ T cells *in vivo*, $CD44^{high}$ T cells from Egr-2 cKO mice proliferated more than $CD44^{high}$ cells from Egr-$2^{loxP/loxP}$ mice (Figure 3.14), indicating that the hyperproliferation of $CD44^{high}$ T cells from Egr-2 cKO mice is driven by an intrinsic mechanism.

These results demonstrate that Egr-2 is involved in controlling the proliferation of $CD44^{high}$ effector T cells *in vivo*.

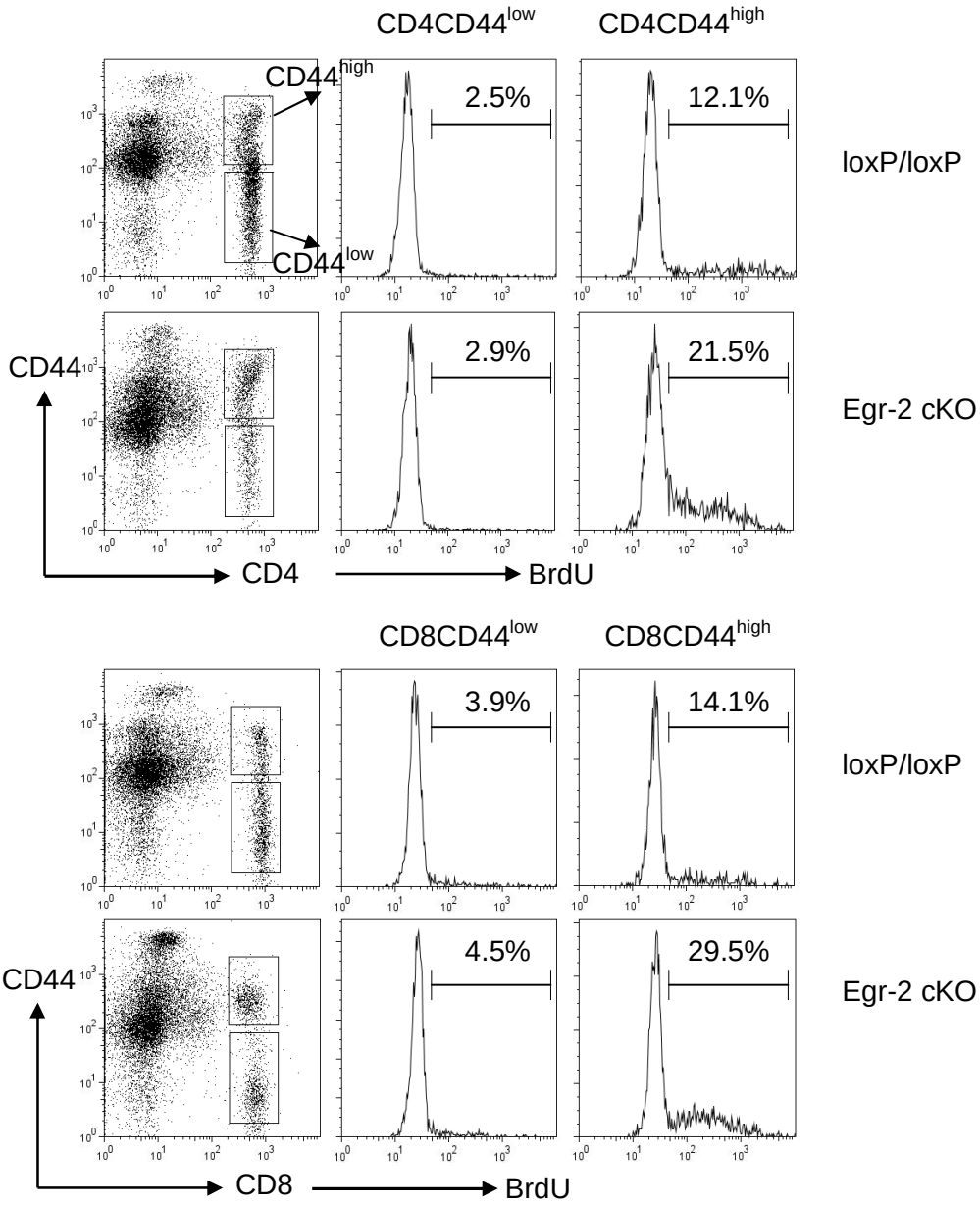

Figure 3.13: Hyper-proliferation of CD44^{high} T cells in Egr-2 cKO mice. 8 month old Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice were supplied with BrdU in drinking water for 9 days. At the end of BrdU treatment, splenocytes were stained with anti-CD4, anti-CD44 and anti-BrdU (top) or anti-CD8, anti-CD44 and anti-BrdU (bottom) and analysed by flow cytometry. BrdU positive T cells were quantified after gating on CD4^{+}CD44^{high}, CD4^{+}CD44^{low}, CD8^{+}CD44^{high} or CD8^{+}CD44^{low} cells. The data is representative of at least 3 mice of each genotype.

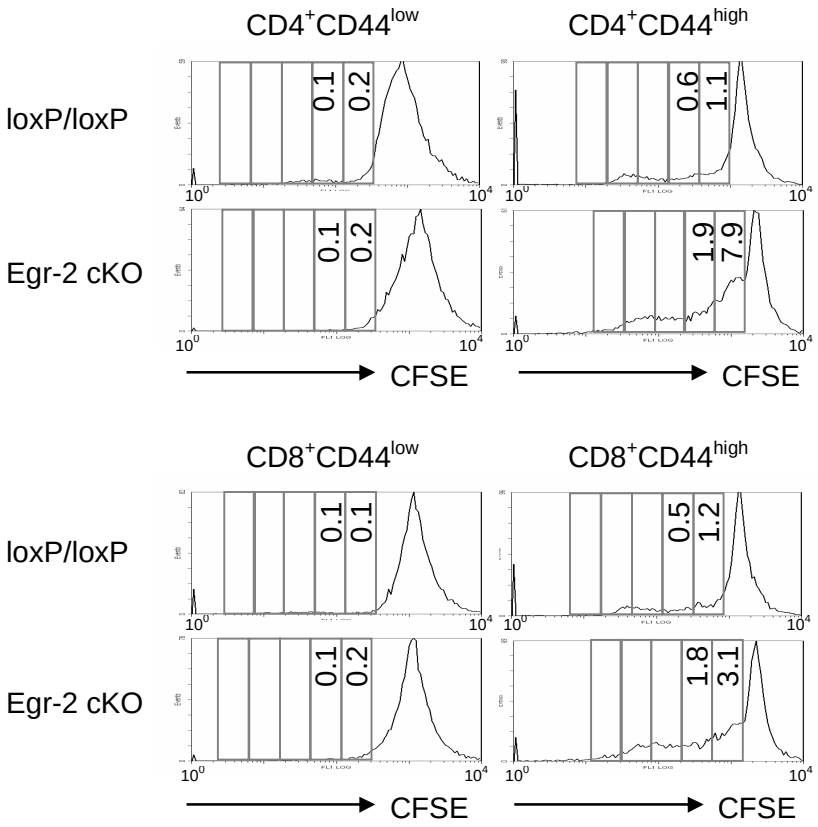

Figure 3.14: Intrinsic hyper-proliferation of CD44^{high} Egr-2 cKO T cells. CD4$^+$ (top) or CD8$^+$ T cells (bottom) were isolated from 7 month old Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice and labelled with CFSE. The labelled cells were adoptively transferred into C57BL/6 mice. Three weeks after transfer, splenocytes were extracted, stained with anti-CD44 and analysed by flow cytometry. Cells were gated on the CD44^{high} or CD44^{low} population and CFSE dilution was analysed. The data is representative of at least 3 mice of each genotype.

3.8 T cells in older Egr-2 cKO mice

To determine what effect the hyper-proliferation of CD44^{high} cells had on the peripheral CD4$^+$ T cell compartment *in vivo,* we examined the splenic CD4$^+$ population at 3, 8 and 15 months of age in Egr-2$^{loxP/loxP}$ and Egr-2 cKO mice. As described above the percentages of CD4$^+$ cells that were CD44^{high} and CD62L^{low}, characteristic of effector phenotype T cells, were slightly increased in 3 month old Egr-2 cKO mice (Figure 3.15,

A). In 8 month old Egr-2 cKO mice, the percentage of $CD44^{high}$ cells was further increased and by 15 months of age all of the $CD4^+$ T cells in Egr-2 cKO mice were $CD44^{high}$ in contrast to ~20% in Egr-$2^{loxP/loxP}$ mice (Figure 3.15, A). CD62L showed a similar, but reversed, pattern with a trend towards expression of low levels of CD62L such that all of the $CD4^+$ T cells were $CD62L^{low}$ in 15 month old Egr-2 cKO mice (Figure 3.15, A). These results indicate that Egr-2 is involved in the control of the homeostasis of the $CD44^{high}$ population *in vivo*.

Indeed, the spleens of 15 month old Egr-2 cKO mice were strikingly enlarged, with a size 5 to 10 times larger than those from age matched Egr-$2^{loxP/loxP}$ mice (Figure 3.15, C). Examination of the cellular composition of these spleens from Egr-2 cKO mice revealed that the $CD4^+$ population was massively increased, more than three fold, compared to age matched Egr-$2^{loxP/loxP}$ mice (Figure 3.15, D). In addition to the expression of high levels of CD44 and low levels of CD62L, these cells in Egr-2 cKO mice also expressed high levels of CD25 and CD69 (Figure 3.15, A). $CD8^+$ cells were also slightly increased in older Egr-2 cKO mice and had a similar increase in cells with an effector phenotype (Figure 3.15, B and D).

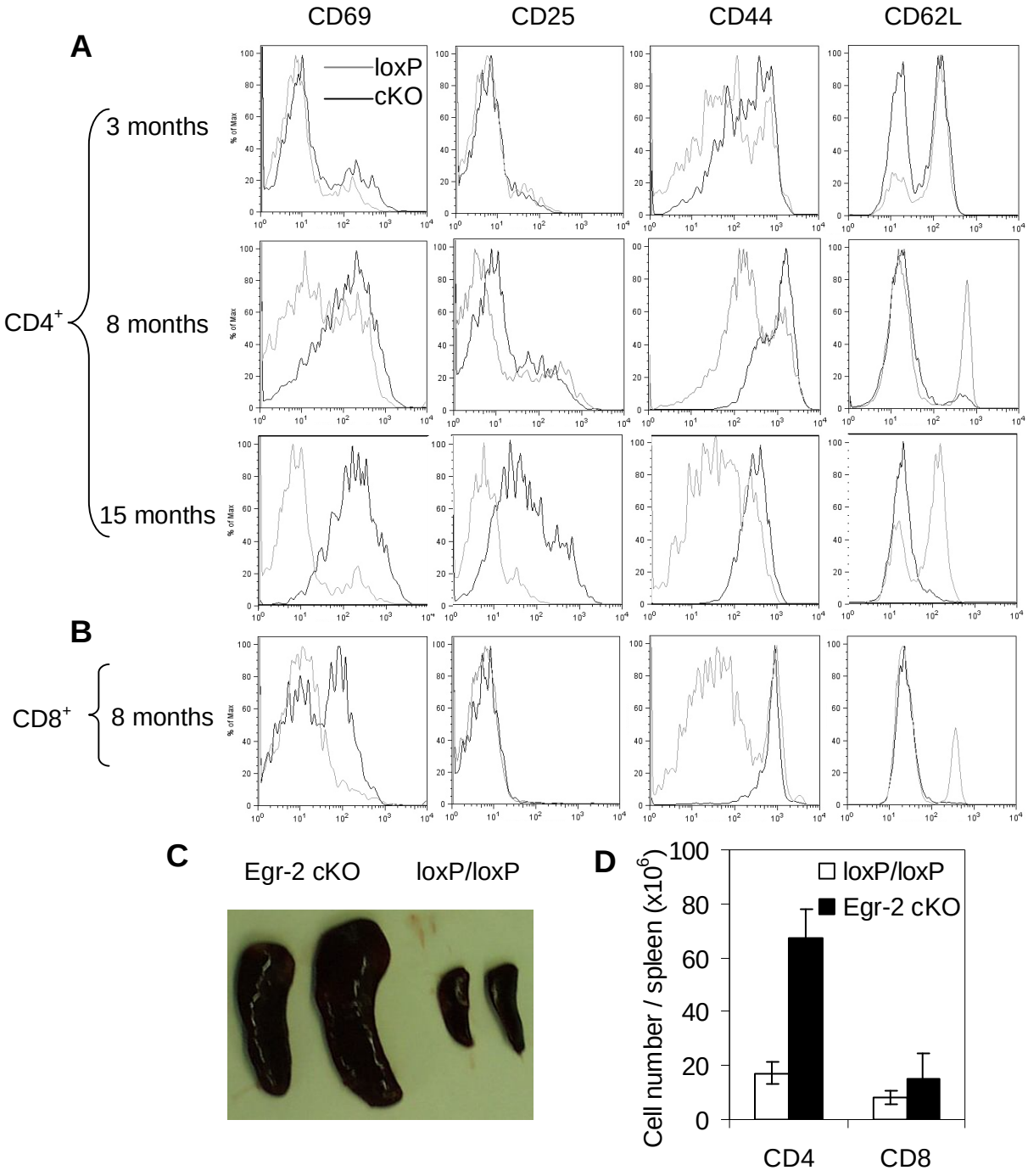

Figure 3.15: Accumulation of effector phenotype T cells in Egr-2 cKO mice. [(**A**) – (**B**)] Splenocytes were extracted Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice of different ages as indicated, stained with the indicated fluorochrome labelled antibodies and analysed by flow cytometry. Expression of CD69, CD25, CD44 and CD62L was examined on gated CD4$^+$ (**A**) or CD8$^+$ (**B**) cells. The data in (**A**) and (**B**) is representative of at least 2 mice of each genotype. (**C**) Spleen size in 15 month old Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice. (**D**) Total number of CD4$^+$ and CD8$^+$ T cells in spleens from older Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice; presented as mean ± standard deviation from 3 mice of each genotype.

3.9 Lupus-like autoimmunity in older Egr-2 cKO mice

In addition to the splenomegaly and lymphoproliferation in older Egr-2 cKO mice, we found that older Egr-2 cKO mice displayed symptoms of a lupus-like autoimmune disease. Egr-2 cKO mice aged more than 1 year displayed hair loss (Figure 3.16, A) and became lethargic. By 15 months of age more than half the Egr-2 cKO mice had died (12 out of 20 mice), whereas no death was observed in age matched Egr-$2^{loxP/loxP}$ mice. Histological examination revealed an accumulation of mononuclear cells in the liver and kidney (Figure 3.16, B). Staining of these sections with antibodies against B and T cell markers revealed that the majority of the cells were T cells with B cells accounting for a small percentage of the cells (Figure 3.16, F). Closer examination of the histological sections revealed the presence of glomerular hyperplasia while immunohistochemistry detected the deposition of immune complexes in the glomeruli (Figure 3.16, D and E). In addition to these signs of glomerulonephritis, protein was detected in the urine of Egr-2 cKO mice (Figure 3.16, C). The total serum Ig was increased in Egr-2 cKO mice to around 3 times the level observed in Egr-$2^{loxP/loxP}$ mice (Figure 3.17, A). Inspection of the Ig subclasses revealed that only the T_H1 dependent IgG2a subclass was significantly increased (Figure 3.17, A), approximately 5 fold, in Egr-2 cKO mice compared to Egr-$2^{loxP/loxP}$ mice. Importantly, we detected auto-antibodies directed against the nuclear components histone and dsDNA in Egr-2 cKO mice (Figure 3.17, B); a hallmark of lupus. The titre of auto-antibodies against histone and dsDNA increased with age and paralleled the progressive accumulation of $CD4^+CD44^{high}$ T cells (Figure 3.15, A). In Egr-2 cKO mice aged 3 months, the percentage of $CD44^{high}$ T cells in the spleen was only slightly increased and the levels of anti-dsDNA and -histone auto-antibodies were normal. Auto-antibodies were detected

after 6 months and this was further increased in 12 month old mice (Figure 3.17, B), mirroring the progressive accumulation of activated $CD44^{high}$ effector T cells (Figure 3.15). Disease incidence was similar in male and female Egr-2 cKO mice (data not shown). Based upon these clinical, histological and serological findings these Egr-2 cKO mice appear to have an autoimmune disease similar to lupus. Thus, Egr-2 deficiency in T cells leads to the loss of self tolerance and the development of a lupus-like autoimmune disease.

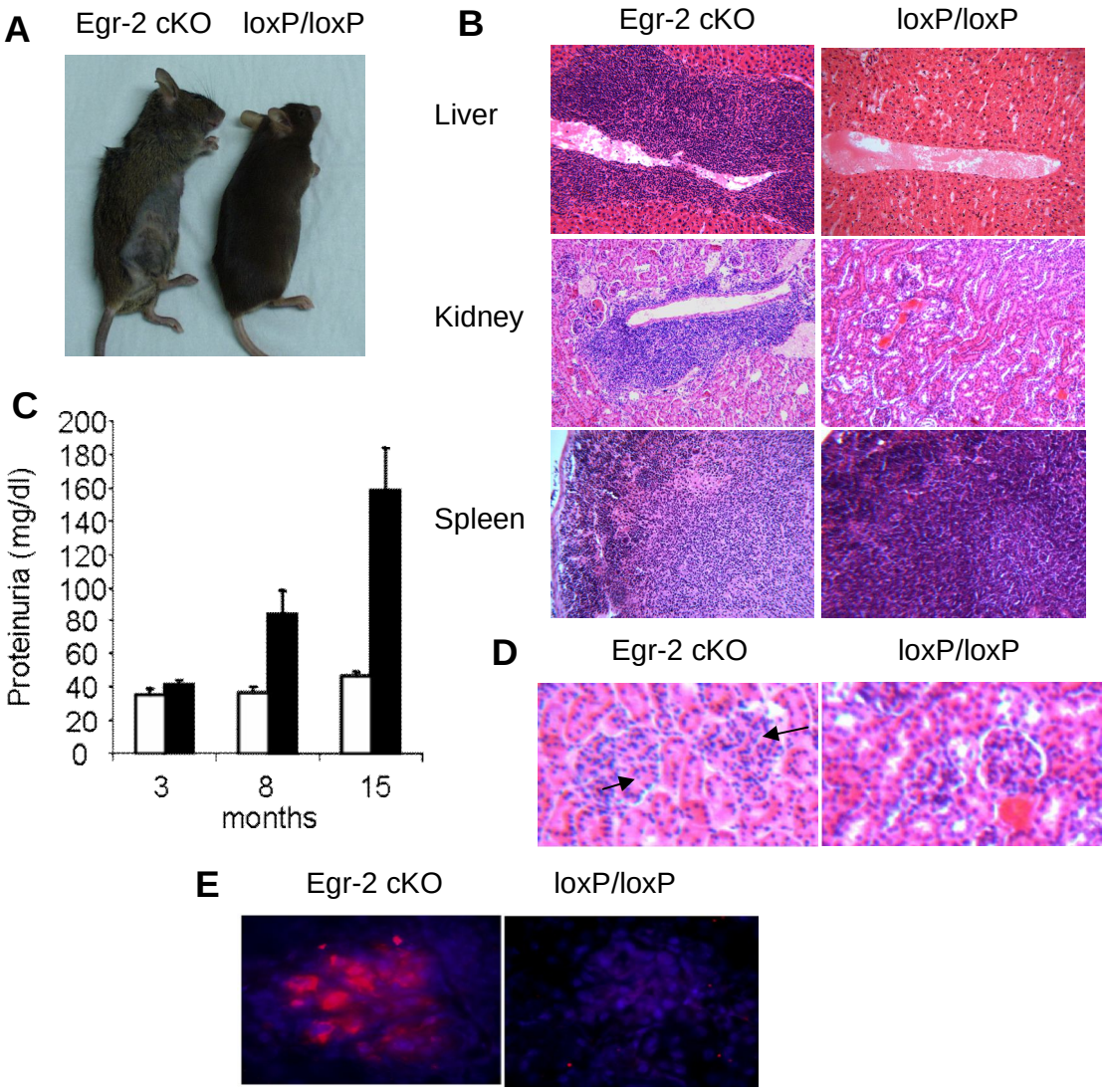

Figure 3.16: Lupus-like autoimmunity in older Egr-2 cKO mice. (**A**) Skin lesions in 15 month old Egr-2 cKO mice compared to age-matched Egr-2$^{loxP/loxP}$ mice. (**B**) Photomicrographs of spleen, liver and kidney tissues from these mice (×10) stained with haematoxylin-eosin (H-E). (**C**) Proteinuria in age-matched Egr-2$^{loxP/loxP}$ and Egr-2 cKO mice. Data is the mean ± standard deviation of values from 10 mice per group. (**D**) Glomeruli of a 15-month old Egr-2 cKO mouse and an aged-matched Egr-2$^{loxP/loxP}$ mouse. (**E**) Immune complex deposition in glomeruli of 15-month old Egr-2 cKO but not in age-matched Egr-2$^{loxP/loxP}$ mice as detected by fluorescently labelled anti-mouse Ig (red). The glomeruli were visualised by DAPI counterstaining in (**E**). (Figure continued overleaf).

F

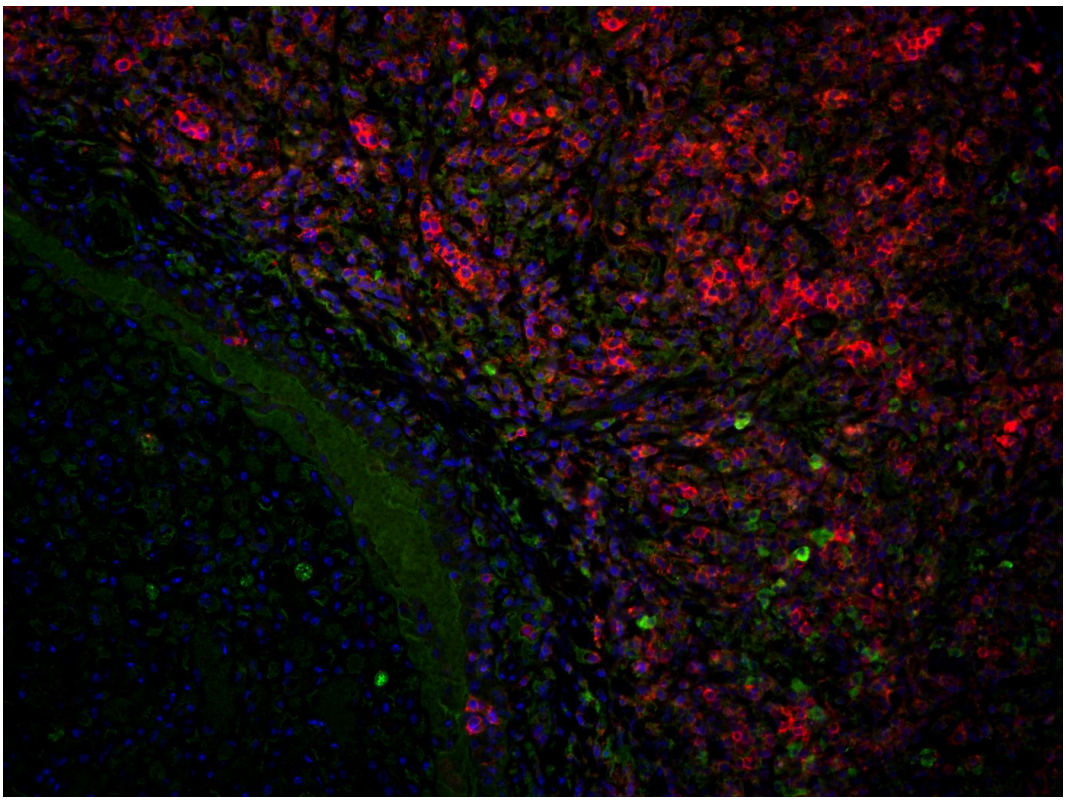

Figure 3.16: Lupus-like autoimmunity in older Egr-2 cKO mice (cont'd). (**F**) Infiltration of T cells in kidney sections of Egr-2 cKO mice. Kidney tissue sections from Egr-2 cKO mice were stained with antibodies against CD3 (red) and B220 (green). The sections were counterstained with DAPI for nuclear staining.

3.10 Auto-antibody production is T cell dependent

In light of the normal expression of Egr-2 in B cells from Egr-2 cKO mice (Figure 3.2, D) and the lack of accumulation of B cells in inflamed tissues (Figure 3.16, F), we speculated that the $CD4^+$ T cells may be driving the development of autoimmunity in Egr-2 cKO mice. To investigate this, Egr-2 cKO $CD4^+$ T cells were isolated, mixed with Egr-$2^{loxP/loxP}$ T cell depleted splenocytes and transferred into RAG-$2^{-/-}$ mice. As a control, Egr-$2^{loxP/loxP}$ $CD4^+$ T cells were isolated, mixed with Egr-2 cKO T cell depleted splenocytes and transferred into RAG-$2^{-/-}$ mice. 75 days later, auto-antibodies against histone and dsDNA were detected in the sera of mice that received Egr-2 cKO $CD4^+$ T cells and Egr-$2^{loxP/loxP}$ T cell depleted splenocytes, but not those that received Egr-$2^{loxP/loxP}$ $CD4^+$ T cells and Egr-2 cKO T cell depleted splenocytes (Figure 3.17, C). These results demonstrate that Egr-2 cKO $CD4^+$ T cells can direct the production of auto-antibodies by Egr-$2^{loxP/loxP}$ B cells and indicate that Egr-2 deficiency in $CD4^+$ T cells is sufficient to initiate auto-antibody production and, presumably, autoimmunity.

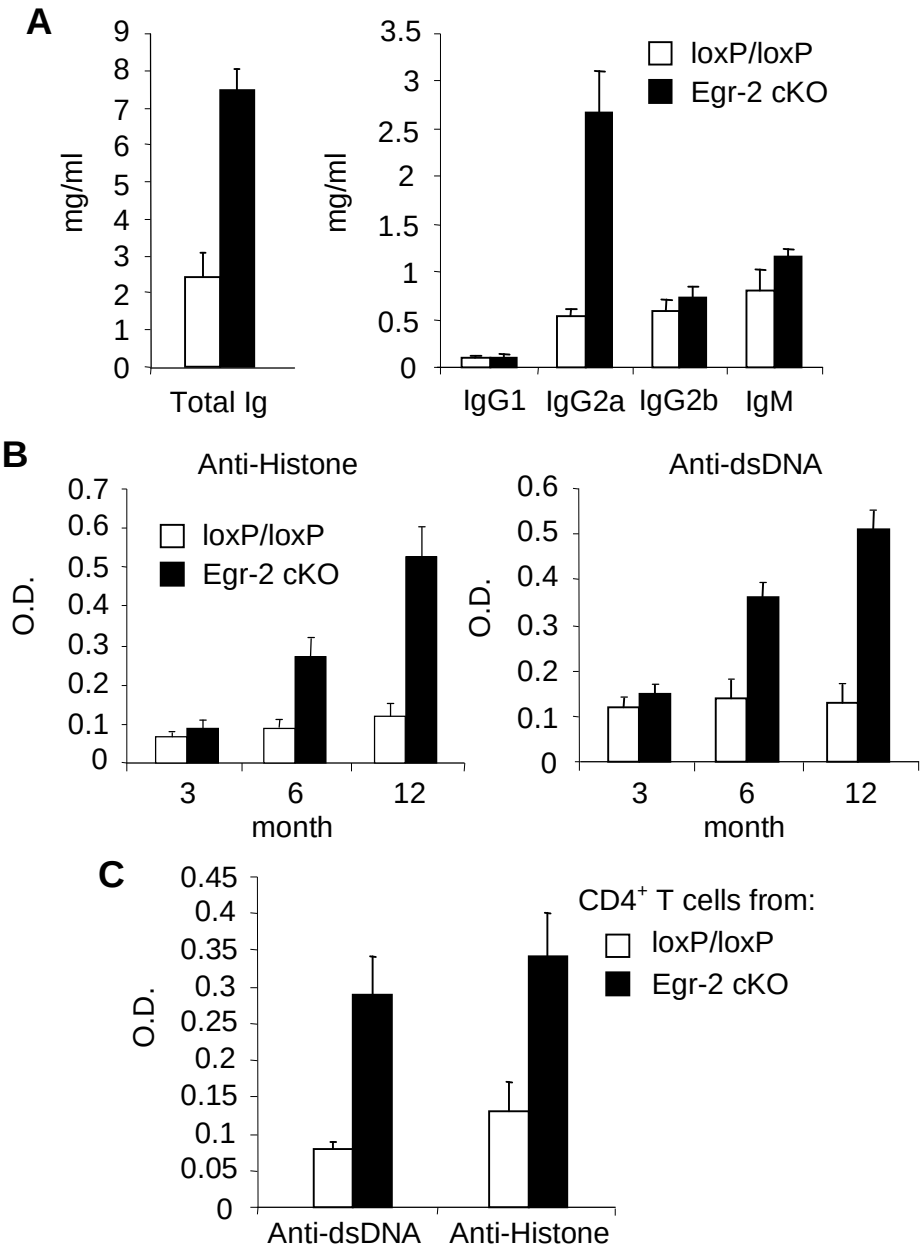

Figure 3.17: Development of autoantibodies in Egr-2 cKO mice. All the antibodies were measured by ELISA and presented either as mg/ml or absorbance (O.D.). (**A**) Serum concentration of total Ig and Ig isotypes in Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice. Data represent the mean ± standard deviation, n = 24 aged 6 - 12 months. (**B**) Serum concentration of IgG antibodies against dsDNA and histone in age matched Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice (n = 6 per group). (**C**) Serum concentration of IgG antibodies against dsDNA and histone in RAG-$2^{-/-}$ mice that were recipients of $CD4^+$ T cells from Egr-2 cKO and T cell-depleted splenocytes from Egr-$2^{loxP/loxP}$ mice or $CD4^+$ T cells from Egr-$2^{loxP/loxP}$and T cell-depleted splenocytes from Egr-2 cKO (n = 5 per group).

3.11 Egr-2 and p21cip1

Previous work by our group found that transfection of Egr-2 into the T cell hybridoma cell line MF2.2D9 resulted in an increase in the expression of the cyclin-dependent kinase inhibitor protein (CDKI) p21cip1 (Anderson *et al.*, 2006); a negative regulator of cell cycle progression (see Sherr and Roberts, 1999). p21cip1 KO mice on a mixed 129/Sv × C57BL/6 background develop a lupus-like autoimmune disease suggesting that p21cip1 is involved in tolerance (Balomenos *et al.*, 2000). To investigate whether p21cip1 may play a role in the Tg4 mouse model of tolerance we examined the expression of p21cip1 in the A0, A2, T0 and T2 populations by western blot. We could not detect p21cip1 expression in either A0 or T0 unstimulated populations (Figure 3.18, A). We found that there was very little p21cip1 in A2 cells (Figure 3.18, A), perhaps because of the time point of stimulation (see also below). However, p21cip1 was readily detectable in the T2 population (Figure 3.18, A). This pattern of p21cip1 expression correlates with that of Egr-2; i.e. undetectable in unstimulated populations but increased after antigen stimulation of both activated and tolerant T cells. To investigate whether Egr-2 is necessary for p21cip1 induction, we examined the expression of p21cip1 after TCR stimulation of Egr-2 cKO and Egr-$2^{loxP/loxP}$ T cells by western blot and Real Time PCR. In contrast to Egr-$2^{loxP/loxP}$ T cells, which strongly upregulated p21cip1 protein after TCR stimulation, Egr-2 cKO T cells showed only a weak induction of p21cip1 protein (Figure 3.18, B). Thus, Egr-2 is necessary for induction of p21cip1 protein expression after TCR engagement. However, when we examined the expression of p21cip1 at the mRNA level 6 hours after TCR engagement we could not detect any difference between Egr-$2^{loxP/loxP}$ and Egr-2 cKO T cells (Figure 3.18, C). Since Egr-2 is expressed rapidly after stimulation we speculated that we might see a difference at an earlier time point. Indeed, Egr-$2^{loxP/loxP}$ T cells stimulated for 2 hours had a 3 fold

increase in expression of p21cip1 compared to Egr-2 cKO T cells stimulated for 2 hours (Figure 3.18, C). Thus, the peak of p21cip1 mRNA expression observed 2 hours after TCR stimulation of Egr-$2^{loxP/loxP}$ T cells is absent in the Egr-2 cKO T cells. These data indicate that the immediate induction of p21cip1 after TCR stimulation is, at least partly, dependent upon Egr-2.

Given that Egr-2 is expressed in $CD44^{high}$ T cells (Figure 3.12), and that Egr-2 deficiency leads to hyper-proliferation and accumulation of $CD44^{high}$ T cells *in vivo* (Figures 3.13, 3.14 and 3.15), we considered the possibility that Egr-2 may be involved in the expression of p21cip1 in $CD44^{high}$ T cells. To investigate this, we examined the expression of p21cip1 in $CD4^{+}CD25^{-}CD44^{high}$ and $CD4^{+}CD25^{-}CD44^{low}$ T cells from Egr-$2^{loxP/loxP}$ and Egr-2 cKO mice. We found that the $CD44^{high}$ population expressed approximately 5 fold higher levels of p21cip1 than the $CD44^{low}$ population in Egr-$2^{loxP/loxP}$ mice (Figure 3.18, D). This pattern of p21cip1 expression correlates with that of Egr-2 in Egr-$2^{loxP/loxP}$ mice (Figure 3.12). In contrast, the $CD44^{high}$ population in Egr-2 cKO mice had more than a 3 fold reduction in expression of p21cip1 compared to Egr-$2^{loxP/loxP}$ $CD44^{high}$ cells (Figure 3.18, D). These results indicate that Egr-2 is involved in the expression of p21cip1 by $CD44^{high}$ T cells and, taken together with the expansion of this population in Egr-2 cKO mice *in vivo*, suggests that Egr-2 may be involved in the control of the homeostasis of $CD44^{high}$ T cells by the induction of p21cip1.

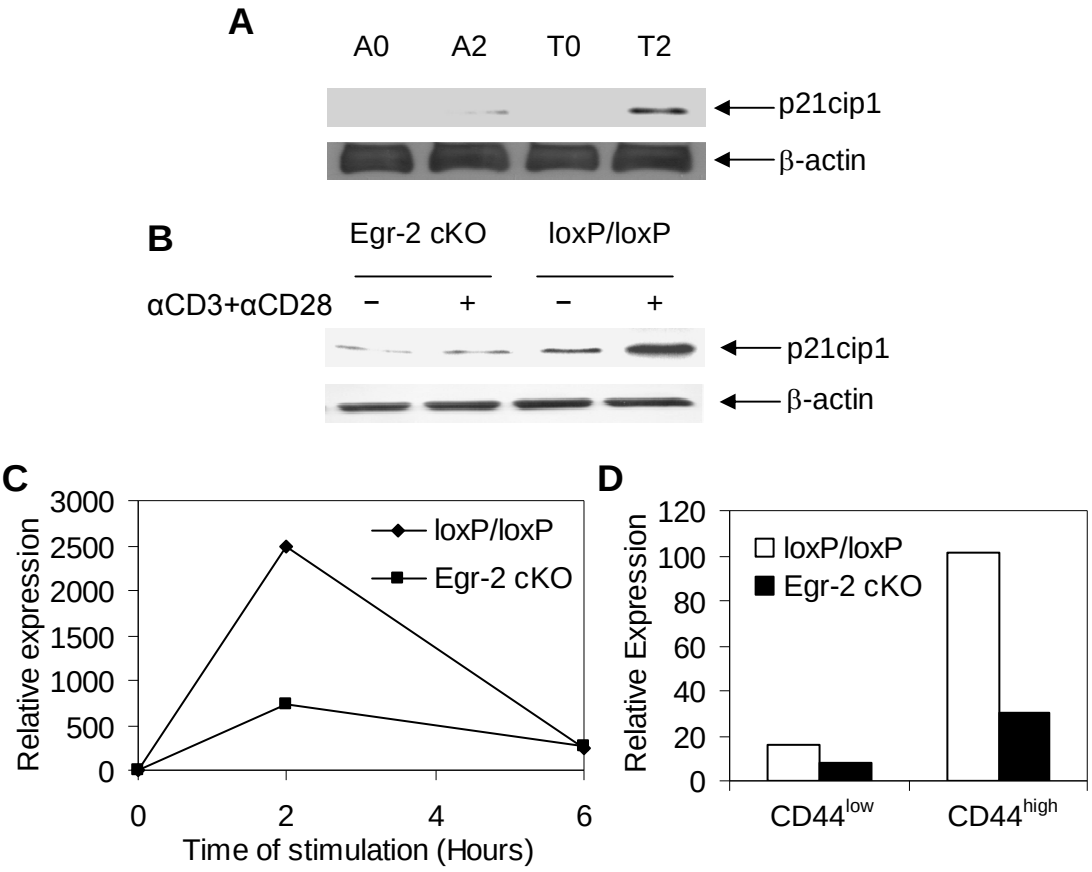

Figure 3.18: Expression of p21cip1 in tolerant and Egr-2 cKO T cells. (**A**) Transgenic Tg4 T cells were tolerised by 10 i.n. administrations of 100µg of the Ac1-9[4Y] peptide from myelin basic protein as described (Anderson *et al.*, 2006). Resting (A0 and T0) and *in vivo* stimulated (A2 and T2) naïve (A) and tolerant (T) $CD4^+$ Tg4 T cells were isolated and p21cip1 expression examined by western blot (**A**). [(**B**) and (**C**)] Splenic $CD4^+$ cells were isolated from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice and stimulated for the indicated time periods (**C**) or for 16 hours (**B**). p21cip1 expression was analysed by western blot (**B**) and Real Time PCR (**C**). (**D**) $CD4^+CD25^-CD44^{low}$ and $CD4^+CD25^-CD44^{high}$ T cells were isolated from 8 month old Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice by FACS and p21cip1 expression examined by Real Time PCR.

Since Egr-2 is a transcription factor, and given the kinetics of Egr-2 and p21cip1 expression, we hypothesised that Egr-2 might directly regulate the expression of p21cip1. To investigate this we examined the 10kb directly upstream of the transcriptional start site of the p21cip1 gene, as defined in Ensembl, for the presence of potential Egr-2 binding sites. We found a sequence similar to the Egr-2 “consensus” site

defined by Swirnoff and Milbrandt (1995); nucleotides -56 to -48 relative to the transcriptional start site (Figure 3.19, A). Notably, this sequence GAGGGGGCG has G residues at the 1, 3, 5, 6, 7 and 9 positions which are important contacts in the crystal structure of DNA bound Egr-1 (Pavletich and Pabo, 1991); the closest homologue to Egr-2 in terms of primary structure. To investigate whether Egr-2 can bind to this site an EMSA was performed. MF2.2D9 cells express Egr-2 after stimulation with anti-CD3 and anti-CD28 coated beads (Figure 3.19, D). Nuclear proteins from stimulated, but not unstimulated, MF2.2D9 cells bound to a nucleotide probe corresponding to this site from the p21cip1 promoter (Figure 3.19, B lanes 1 and 2). This complex could be supershifted by the addition of an antibody against Egr-2 but not by an anti-Egr-3 antibody (Figure 3.19, B lanes 3 and 4). To determine whether this is relevant *in vivo*, a ChIP assay was performed. We found that the p21cip1 promoter could be immunoprecipitated from Egr-2 transduced cells by anti-Egr-2, but not irrelevant control, antibody (Figure 3.19, C). The lack of immunoprecipitation from EGFP transduced control cells, in which Egr-2 is not expressed (Figure 3.19, D), confirms the specificity of immunoprecipitation by the anti-Egr-2 antibody (Figure 3.19, C). In contrast, the SOCS3 CDS was not detected in anti-Egr-2 immunoprecipitates from Egr-2 transduced cells by PCR (Figure 3.19, C). These data demonstrate that, at least in MF2.2D9 cells, Egr-2 directly binds to the p21cip1 promoter. Taken together with the results from Egr-2 cKO T cells, these data suggest that Egr-2 directly regulates p21cip1 expression immediately after TCR engagement and in $CD44^{high}$ T cells. The correlation of Egr-2 and p21cip1 expression in Tg4 mice, and the autoimmune syndrome in Egr-2 cKO mice, suggests that Egr-2 mediated induction of p21cip1 may be involved in maintaining self tolerance.

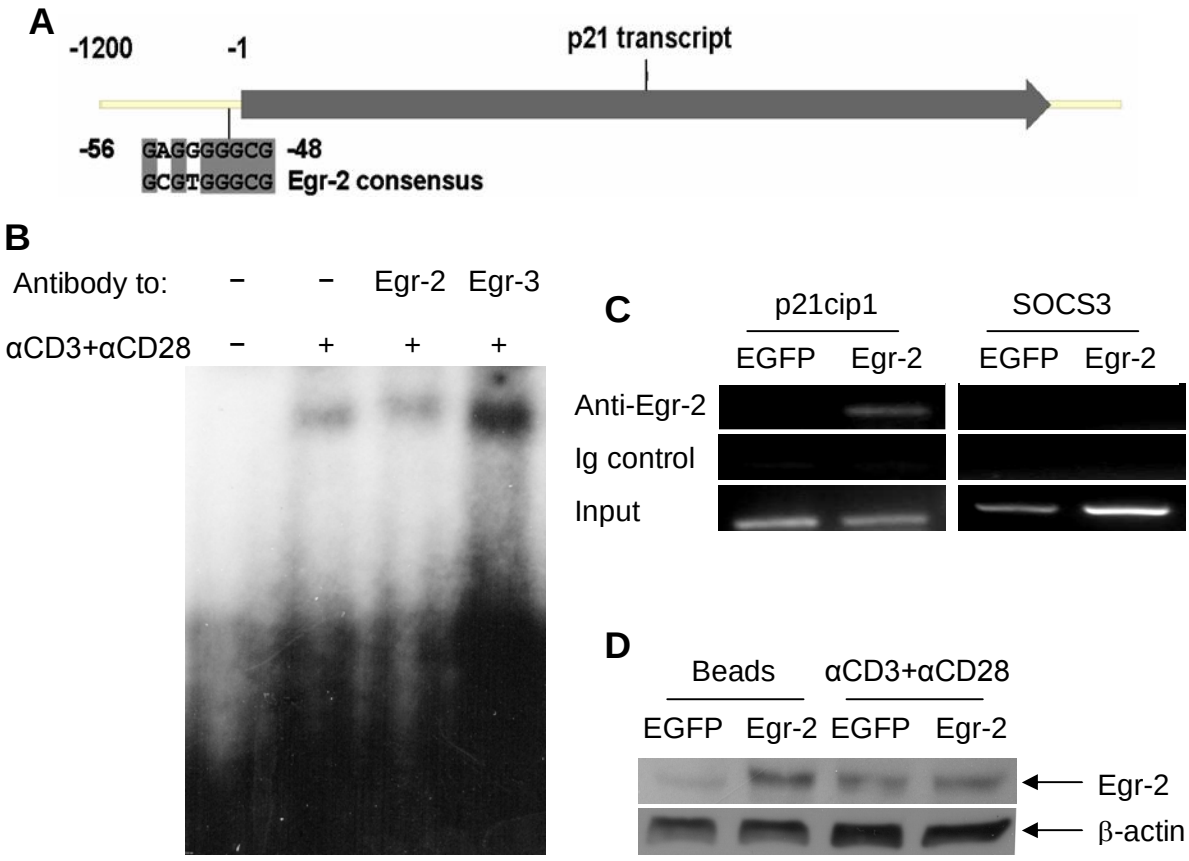

Figure 3.19: Interaction of Egr-2 with the p21cip1 promoter *in vitro* and *in vivo*. (**A**) Identification of a potential Egr-2 binding site in the proximal promoter region of the p21cip1 gene. (**B**) Electrophoretic mobility-shift assay to evaluate the binding of Egr-2 to oligonucleotides corresponding to this sequence from the p21cip1 promoter. Nuclear extracts from stimulated and unstimulated MF2.2D9 cells were incubated with radioactively labelled oligonucleotides and complexes were resolved by native gel electrophoresis. Where indicated anti-Egr-2 or anti-Egr-3 antibody was added. (**C**) Chromatin immunoprecipitation assay. EGFP or Egr-2 transduced MF2.2D9 T cells were crosslinked with formaldehyde and chromatin immunoprecipitated with anti-Egr-2 or irrelevant control antibody. The immunoprecipitated DNA was purified and used as a template for PCR. (**D**) EGFP and Egr-2 transduced cells were stimulated with anti-CD3 and anti-CD28 for 16 hours or left unstimulated. Expression of Egr-2 was analysed by western blot.

3.12 Microarray analysis of Egr-2 cKO T cells

While defective expression of p21cip1 provides a plausible explanation for the hyper-proliferation and accumulation of effector T cells *in vivo*, whether defective expression of p21cip1 can fully explain the loss of tolerance in Egr-2 cKO mice is unclear. Since Egr-2 is a transcription factor it could potentially regulate hundreds of genes, any number of which might be involved in the maintenance of tolerance. Thus, there are likely to be other genes regulated by Egr-2 that are relevant to autoimmune disease development in Egr-2 cKO mice. To examine the effects of Egr-2 in naïve and activated T cells, we assessed the genome-wide transcriptional profiles of unstimulated and anti-CD3 stimulated Egr-$2^{loxP/loxP}$ and Egr-2 cKO $CD4^+$ T cells using Illumina MouseRef-8 BeadChip expression arrays. These arrays consist of 50-mer oligonucleotide probes directed against ~24,000 well annotated RefSeq transcripts. $CD4^+$ T cells from 3 month old Egr-$2^{loxP/loxP}$ and Egr-2 cKO mice, which have similar percentages of $CD4^+CD44^{high}$ T cells (Figure 3.7, E), were used in order to try to minimise complications from the increased percentage of effector T cells in Egr-2 cKO mice. $CD4^+$ T cells were isolated, stimulated with anti-CD3 or left unstimulated, and RNA was extracted and purified as described in the methods section. RNA labelling, array scanning and data normalisation was performed by the Genome Centre, Queen Mary, University of London.

Following receipt of the normalised data, we first examined the expression of several well characterised T cell markers on the array. CD3ε was expressed at similar levels in Egr-$2^{loxP/loxP}$ and Egr-2 cKO $CD4^+$ T cells (Figure 3.20). Furthermore, markers of activated T cells such as CD44, CD25 and CD69 were upregulated normally in Egr-2 cKO cells in response to TCR stimulation with an expression level similar to Egr-$2^{loxP/loxP}$ $CD4^+$ T cells (Figure 3.20). Consistent with the previous findings, Egr-2 was undetectable in unstimulated Egr-2 cKO $CD4^+$ T cells and only very weak expression

was detected in stimulated Egr-2 cKO $CD4^+$ T cells (Figure 3.20). These data indicate that the T cells were successfully activated and that the cells were effectively Egr-2 null as expected.

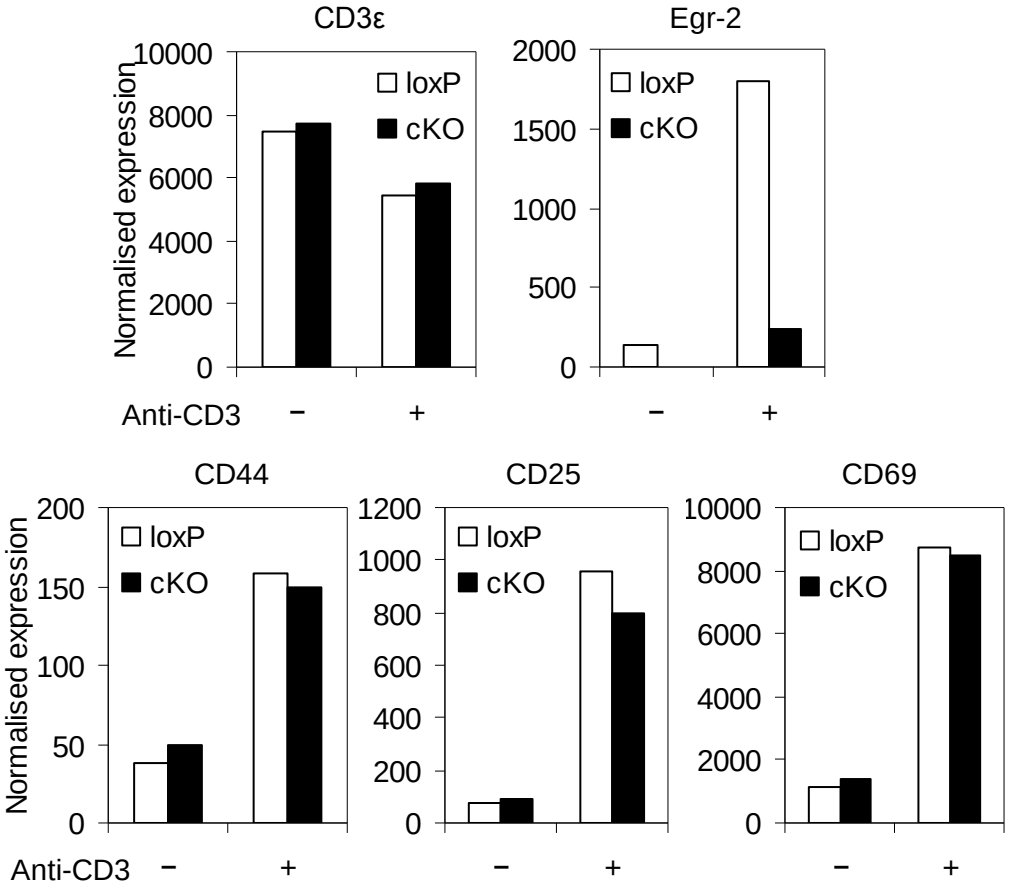

Figure 3.20: Microarray results for well characterised T cell markers and Egr-2. Splenic $CD4^+$ cells were isolated from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice, stimulated with anti-CD3 for 6 hours and global gene expression profiles analysed by microarray. Normalised expression on the microarray is shown. Where the gene was present more than once (CD44, CD69), the entry with the highest expression is presented.

Egr-1 was expressed at similar levels in Egr-$2^{loxP/loxP}$ and Egr-2 cKO $CD4^+$ T cells, while Egr-3 and Egr-4 expression could barely be detected (Figure 3.21). The low expression of Egr-3 may be due to the time point examined since Powell and colleagues found expression of Egr-3 mRNA only at very early time points after TCR engagement (Safford *et al.*, 2005). In any case, these results suggest that there is no compensatory

up-regulation of other Egr family members in the Egr-2 cKO T cells at least at this time point.

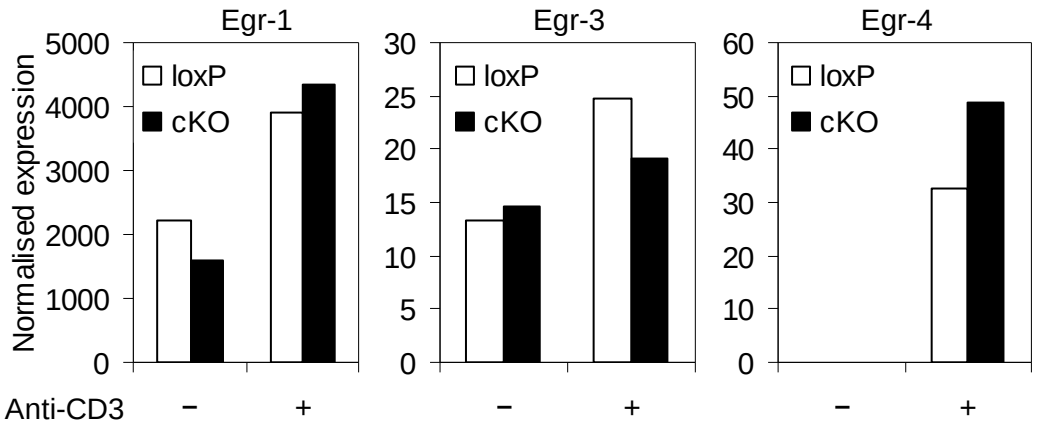

Figure 3.21: Microarray results for other Egr family genes. Splenic $CD4^+$ cells were isolated from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice, stimulated with anti-CD3 for 6 hours and global gene expression profiles analysed by microarray. Normalised expression on the microarray is shown.

To validate the microarray results, Real Time RT-PCR was performed. Thirty genes were validated by Real Time RT-PCR (Figures 3.22-3.28 and data not shown); of these only two did not correlate with the microarray (data not shown). These two genes both had low intensity of less than 20 (a detection p-value between 0.05 and 0.01) on the microarray suggesting that perhaps our criteria for selection should have been more stringent and that a signal intensity of less than 20 (a detection p-value greater than 0.01) should be considered background. While the patterns of expression usually correlated fairly well with those from the microarray, the relative levels of expression sometimes differed. For example, Figure 3.22 compares the expression of IL-2 and IFN-γ in unstimulated and stimulated Egr-2 cKO and Egr-$2^{loxP/loxP}$ $CD4^+$ T cells as detected by the two methods. Both methods showed similar patterns of cytokine expression but the microarray showed only a slight increase in the expression of IFN-γ while Real Time PCR found a 2 fold increase in expression of this cytokine in Egr-2 cKO $CD4^+$ T

cells compared to Egr-2$^{loxP/loxP}$ cells. This quantitative difference may be due to the different sensitivity of the two techniques.

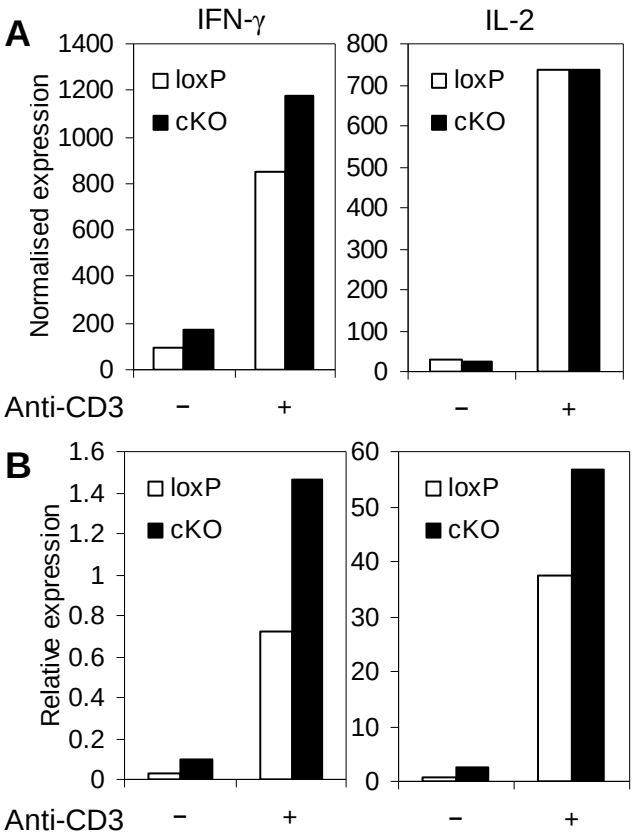

Figure 3.22: IL-2 and IFN-γ mRNA expression in Egr-2 cKO CD4$^+$ T cells. Splenic CD4$^+$ cells were isolated from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice and stimulated with anti-CD3 for 6 hours. (**A**) Normalised expression on the microarray (**B**) Real Time PCR validation

The full set of microarray data is available from the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-1698. We focussed on genes that showed a difference of at least three-fold between Egr-2$^{loxP/loxP}$ and Egr-2 cKO CD4$^+$ T cells (see section 2.20). More than 500 genes met these criteria, of these nearly a third were of unknown function. More than a quarter of the remaining genes were metabolic enzymes, more than double any other category, consistent with a previous report from Egr-1, Egr-3 DKO mice (Carter *et al.*, 2007). The next most common categories were cell surface receptors; cytokines, chemokines and growth

factors; signal transduction proteins and enzymes; and transcription factors; with around 50 genes in each category. The remaining genes encoded structural cytoskeletal proteins and ion channels and transport molecules; with around 20 genes in each of these two categories. Some of the largest changes in expression levels were in cytokine genes and other signalling molecules. In particular, pro-inflammatory cytokines were increased while T_H2 cytokines were decreased in stimulated Egr-2 cKO cells compared to stimulated Egr-$2^{loxP/loxP}$ cells. Given their potential relevance to T cell function, many of these genes were selected for Real Time PCR validation.

3.12.1 Genes up-regulated in Egr-2 cKO cells

3.12.1.1 Proinflammatory cytokines and related genes

Inspection of the microarray results revealed that the expression of two of the IL-17 family cytokines was higher in stimulated Egr-2 cKO $CD4^+$ T cells than in stimulated Egr-$2^{loxP/loxP}$ $CD4^+$ T cells. The microarray data showed a five fold increase for IL-17F while IL-17A was increased two fold (Figure 3.23, A). Real Time RT-PCR showed a 3.5 fold increase for IL-17F and a three fold increase for IL-17A confirming the increased expression of these two cytokines (Figure 3.23, B).

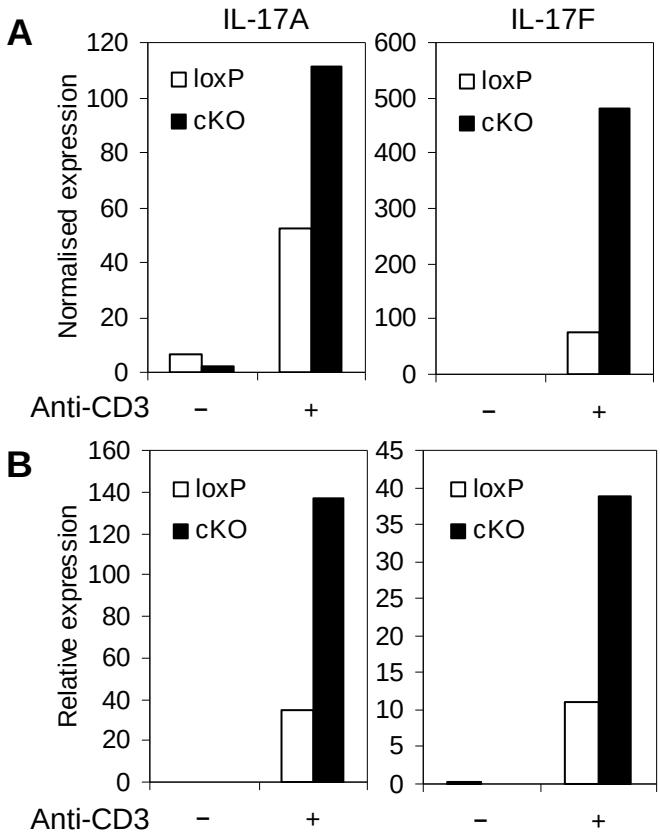

Figure 3.23: IL-17A and IL-17F mRNA expression in Egr-2 cKO CD4$^+$ T cells. Splenic CD4$^+$ cells were isolated from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice and stimulated with anti-CD3 for 6 hours. (**A**) Normalised expression on the microarray (**B**) Real Time PCR validation

Another pro-inflammatory cytokine, IL-1α, was increased 3 fold in stimulated Egr-2 cKO CD4$^+$ T cells compared to stimulated Egr-2$^{loxP/loxP}$ CD4$^+$ T cells according to the microarray (Figure 3.24, A). Real Time RT-PCR found a four fold difference between IL-1α expression in these two populations (Figure 3.24, B).

The microarray also indicated that the Chemokine Receptor CCR6 was increased in stimulated Egr-2 cKO CD4$^+$ T cells (Figure 3.24, A). Real Time RT-PCR confirmed a four fold increase in stimulated Egr-2 cKO CD4$^+$ T cells compared to Egr-2$^{loxP/loxP}$ CD4$^+$ T cells (Figure 3.24, B).

Interestingly, IL-21, which has been reported to be involved in the generation of IL-17 producing $CD4^+$ T cells (Korn *et al.*, 2007; Nurieva *et al.*, 2007; Zhou *et al.*, 2007), was increased in both unstimulated and stimulated $CD4^+$ T cells from Egr-2 cKO mice compared to the corresponding populations from Egr-$2^{loxP/loxP}$ mice, but was significantly higher in unstimulated T cells (Figure 3.24, A). Real Time RT-PCR found that the difference in stimulated cells was only ~2 fold but in unstimulated cells the expression of IL-21 was more than 50 times higher in Egr-2 cKO $CD4^+$ T cells than in Egr-$2^{loxP/loxP}$ $CD4^+$ T cells (Figure 3.24, B). In fact the expression of IL-21 in unstimulated Egr-2 cKO $CD4^+$ T cells was more than three times the expression in stimulated Egr-2 cKO $CD4^+$ T cells (Figure 3.24, B).

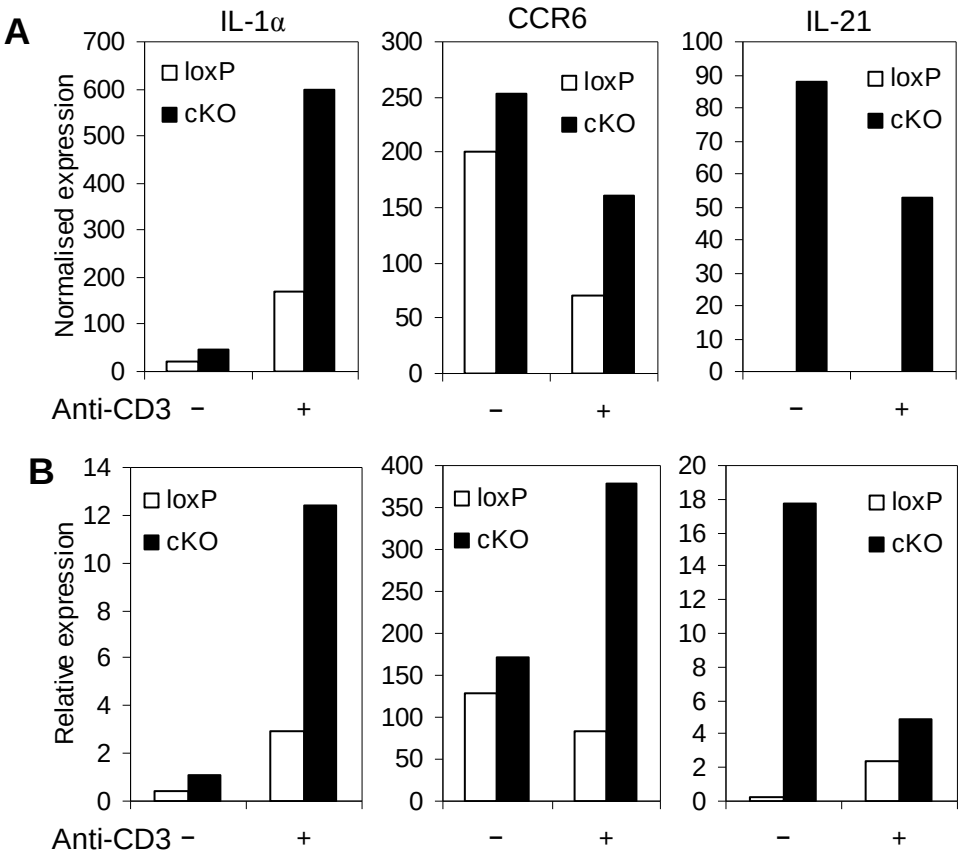

Figure 3.24: IL-1α, CCR6 and IL-21 mRNA expression in Egr-2 cKO $CD4^+$ T cells. Splenic $CD4^+$ cells were isolated from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice and stimulated with anti-CD3 for 6 hours. (**A**) Normalised expression on the microarray (**B**) Real Time PCR validation

3.12.1.2 Anti-inflammatory and pro-homeostatic cytokines

Interestingly, the anti-inflammatory cytokine IL-10 was increased in both unstimulated and stimulated $CD4^+$ T cells from Egr-2 cKO mice compared to the corresponding populations from Egr-$2^{loxP/loxP}$ mice (Figure 3.25). In stimulated cells the difference was around three fold, as determined by both microarray and Real Time RT-PCR, but in unstimulated cells the difference was more than seven times (Figure 3.25).

Intriguingly, the microarray indicated that Egr-2 cKO T cells expressed increased levels of the IL-2 related cytokine IL-15 (Figure 3.25, A). Real Time PCR confirmed that IL-15 was increased around 2.5 times in stimulated $CD4^+$ T cells from Egr-2 cKO mice compared to stimulated Egr-$2^{loxP/loxP}$ $CD4^+$ T cells (Figure 3.25, B).

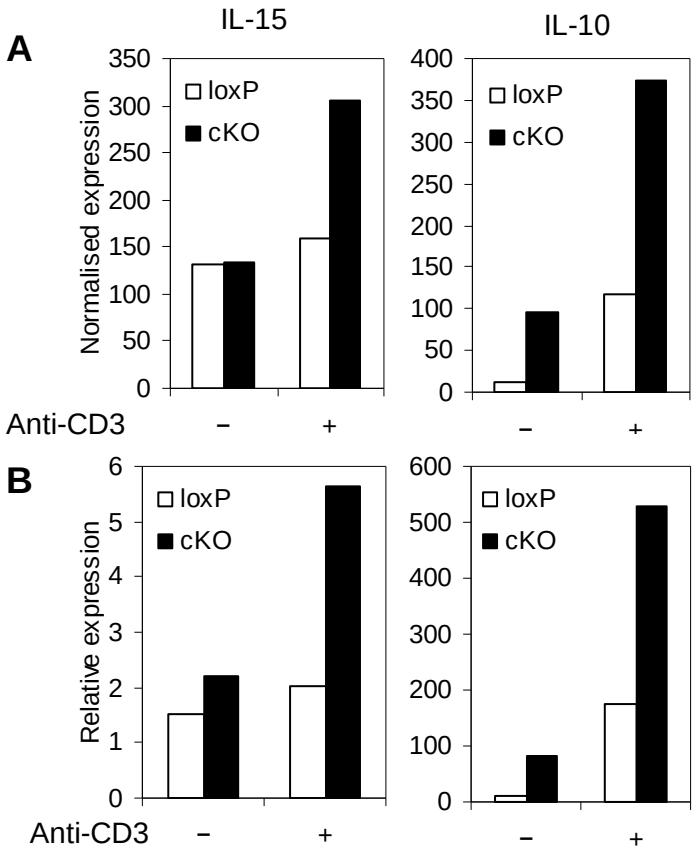

Figure 3.25: IL-15 and IL-10 mRNA expression in Egr-2 cKO CD4$^+$ T cells. Splenic CD4$^+$ cells were isolated from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice and stimulated with anti-CD3 for 6 hours. (**A**) Normalised expression on the microarray (**B**) Real Time PCR validation

3.12.2 Genes down-regulated in Egr-2 cKO cells

3.12.2.1 T_H2 cytokines

The most prominent family of genes that was downregulated more than three-fold was T_H2 cytokines. IL-4, IL-5 and IL-13 were not expressed in the absence of stimulation, while they were reduced at least three fold in Egr-2 cKO CD4$^+$ T cells compared to Egr-2$^{loxP/loxP}$ cells in response to anti-CD3 stimulation according to the microarray (Figure 3.26, A and data not shown). Real Time PCR found that IL-4 was reduced more than

three fold, while IL-13 was reduced more then four fold, in stimulated Egr-2 cKO $CD4^+$ T cells compared to stimulated Egr-$2^{loxP/loxP}$ cells (Figure 3.26, B).

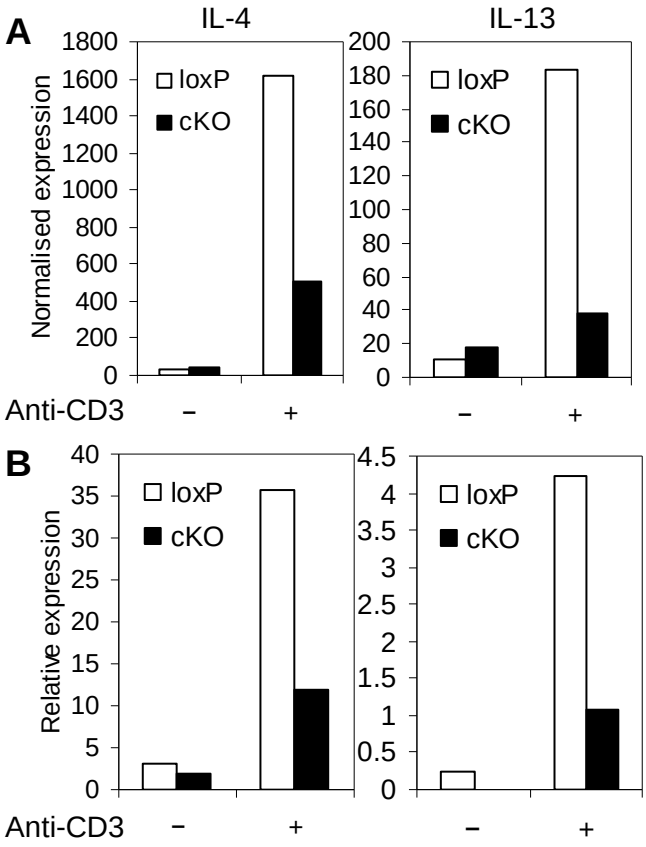

Figure 3.26: IL-4 and IL-13 mRNA expression in Egr-2 cKO $CD4^+$ T cells. Splenic $CD4^+$ cells were isolated from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice and stimulated with anti-CD3 for 6 hours. (**A**) Normalised expression on the microarray (**B**) Real Time PCR validation

3.12.2.2 Anti-apoptotic genes

Although the microarray found only a slight decrease in the expression of the anti-apoptotic gene Bcl-2 (Figure 3.27, A), Real Time PCR found that the expression of this gene was decreased around two fold in stimulated Egr-2 cKO $CD4^+$ T cells compared to stimulated Egr-$2^{loxP/loxP}$ cells (Figure 3.27, B). Bcl-X_L showed a similar expression profile; however, the level of expression was very low (data not shown).

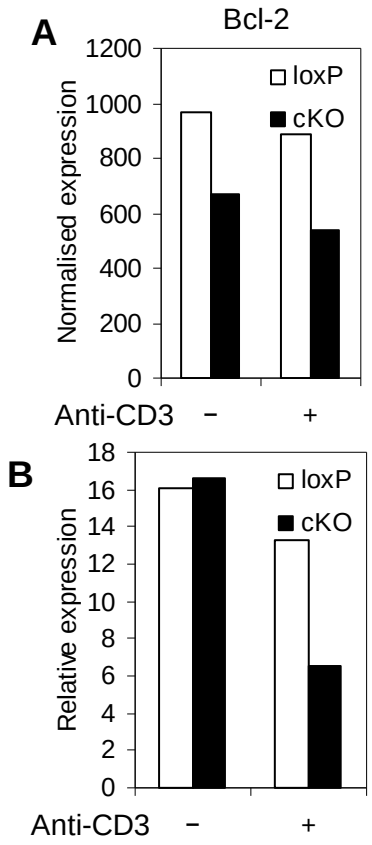

Figure 3.27: Bcl-2 mRNA expression in Egr-2 cKO CD4$^+$ T cells. Splenic CD4$^+$ cells were isolated from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice and stimulated with anti-CD3 for 6 hours. (**A**) Normalised expression on the microarray (**B**) Real Time PCR validation

In summary, we discovered that Egr-2 cKO T cells express increased levels of pro-inflammatory cytokines and decreased levels of T_H2 cytokines.

3.13 Cytokine profile of Egr-2 cKO T cells

We have demonstrated that Egr-2 cKO T cells express increased levels of pro-inflammatory cytokine mRNAs after TCR engagement *in vitro*. To determine whether this reflects increased numbers of IFN-γ and IL-17 producing CD4$^+$ T cells *in vivo*, CD4$^+$ cells were isolated from Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice of different ages, stimulated with PMA and Ionomycin and IFN-γ and IL-17 production was examined by

intracellular cytokine staining. In 3 month old Egr-2$^{loxP/loxP}$ mice, ~2% of CD4^{+} cells produced IFN-γ while IL-17 producing cells could barely be detected (Figure 3.28, A). In contrast, in 3 month old Egr-2 cKO mice more than 5% of CD4^{+} cells produced IFN-γ and 1.5% produced IL-17 (Figure 3.28, A). While the cytokine profile of 8 month old Egr-2$^{loxP/loxP}$ mice was similar to 3 month old Egr-2$^{loxP/loxP}$ mice (Figure 3.28, A), the percentages of IFN-γ and IL-17 producing cells in 8 month old Egr-2 cKO mice were increased compared to 3 month old Egr-2 cKO mice with more than 10% of cells producing IFN-γ and more than 4% of cells producing IL-17 (Figure 3.28, A). Although the percentages of cytokine positive cells was increased in 12 month old Egr-2$^{loxP/loxP}$ mice, to ~5.5% and ~2% for IFN-γ and IL-17 respectively, this was still significantly less than age matched Egr-2 cKO mice in which ~15% were IFN-γ^{+} and ~9% were IL-17^{+} (Figure 3.28, A).

This progressive increase in the percentage of cells producing IFN-γ and IL-17 as the mice age parallels the progressive accumulation of CD44^{high} cells (Figure 3.15, A). To determine whether the CD44^{high} cells in Egr-2 cKO mice are functionally altered, we examined cytokine production by CD4^{+}CD44^{low} and CD4^{+}CD44^{high} cells from 8 month old Egr-2$^{loxP/loxP}$ and Egr-2 cKO mice. We found that there was very little cytokine production by CD4^{+}CD44^{low} cells in either Egr-2 cKO or Egr-2$^{loxP/loxP}$ mice; less than 2% produced IFN-γ and less than 1% produced IL-17 (Figure 3.28, B). However, around 14% of CD4^{+}CD44^{high} cells from Egr-2 cKO mice produced IFN-γ and 7% produced IL-17, compared to less than 4% and 2%, for IFN-γ and IL-17 respectively, of CD4^{+}CD44^{high} cells in Egr-2$^{loxP/loxP}$ mice (Figure 3.28, B). In addition we found that the percentage of CD4^{+} cells that were CD44^{high} IL-4^{+} was reduced in Egr-2 cKO compared to Egr-2$^{loxP/loxP}$ mice (Figure 3.28, C). Furthermore, the percentage of cells producing IL-2 was similar in Egr-2 cKO and Egr-2$^{loxP/loxP}$ mice (Figure 3.28, C) indicating that

the expansion of the $CD4^+CD44^{high}$ T cell population was not due to enhanced autocrine IL-2 signalling. Thus, the $CD4^+CD44^{high}$ T cells in Egr-2 cKO mice are predominately IFN-γ and/or IL-17 producing effector T cells.

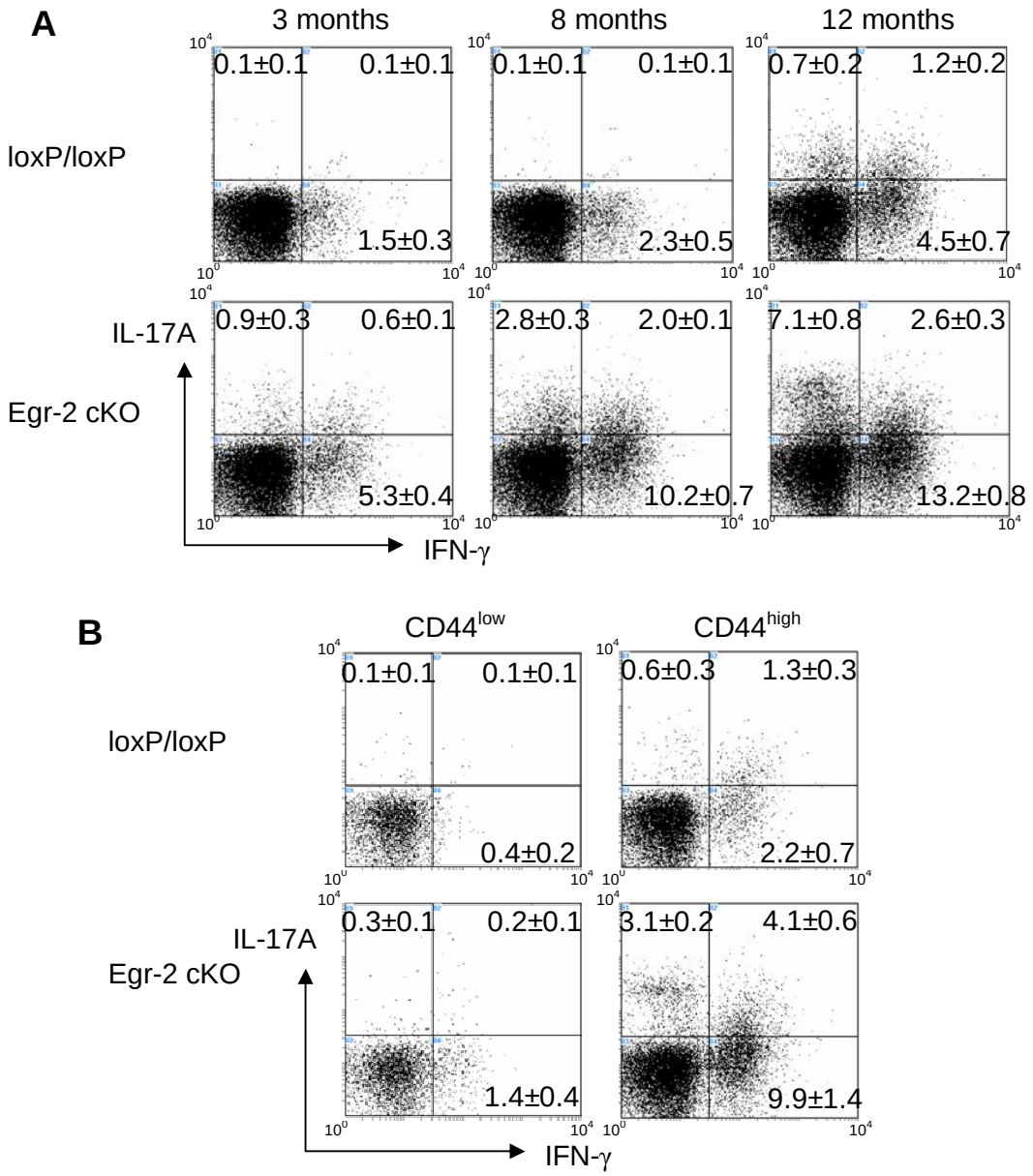

Figure 3.28: Cytokine production by $CD4^+$ T cells in Egr-2 cKO mice. $CD4^+$ T cells [(**A**) and (**B**)] or splenocytes (**C**) were isolated from Egr-2 cKO and Egr-$2^{loxP/loxP}$ mice and stimulated for 5 hours with PMA and Ionomycin and intracellular cytokine staining was performed. (**A**) $CD4^+$ T cells were isolated from mice of different ages as indicated and stained with IFN-γ and IL-17 antibodies. (**B**) $CD4^+$ T cells from 8 month old mice and stained with CD44, IFN-γ and IL-17 antibodies. Cytokine positive cells were quantified after gating for $CD44^{low}$ or $CD44^{high}$. (Figure continued overleaf).

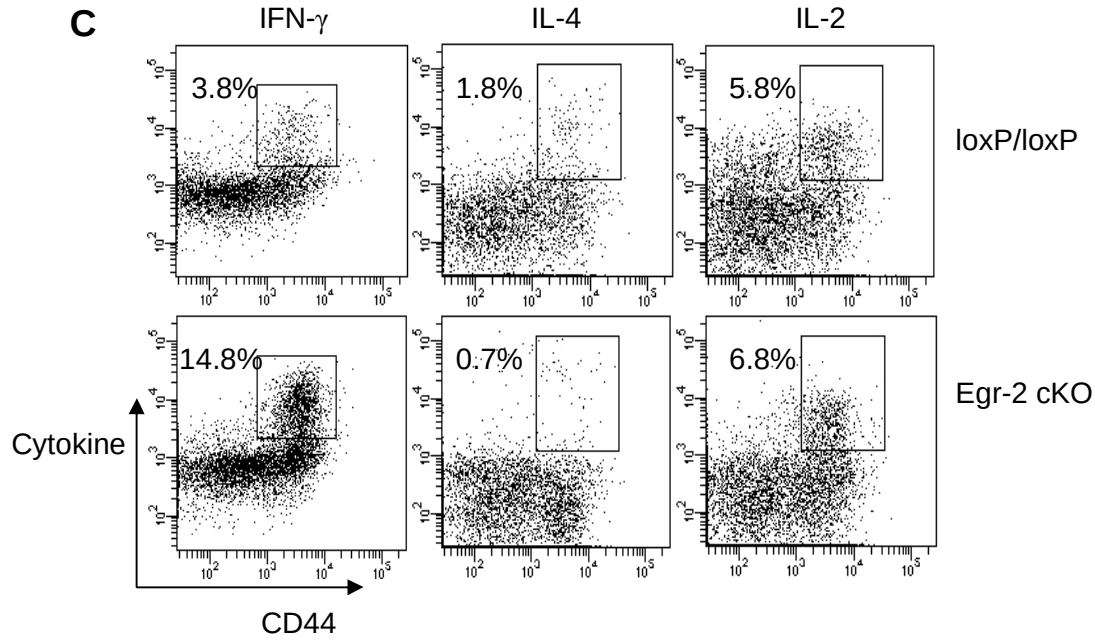

Figure 3.28: Cytokine production by CD4$^+$ T cells in Egr-2 cKO mice (cont'd). (**C**) Splenocytes from 8 month old mice were stained with CD4, CD44 and the indicated cytokine antibodies. Cells were gated on the CD4$^+$ population and CD44^{high} cytokine positive cells were quantified.

Chapter 4: DISCUSSION

We found that Egr-2 is expressed in tolerant T cells after *in vivo* antigen stimulation. On the basis of this finding we generated Egr-2 cKO and Tg mice to examine the roles of Egr-2 in T cells *in vivo*.

4.1 Egr-2 in T cell development

Although Egr-2 is expressed in thymocytes and has been implicated in β-selection (Carleton *et al.*, 2002), we did not detect any defects in T cell development in Egr-2 cKO mice; although we cannot formally exclude the possibility of altered positive or negative selection. The apparently normal thymocyte development suggests that either Egr-2 function at this point is redundant, as has been suggested for Egr-1 and Egr-3 (Carter *et al.*, 2007), or that Cre mediated deletion was incomplete at this stage.

In contrast, Egr-2 Tg mice had profound defects in thymocyte development with accumulation of DN4 and ISP cells; those stages that immediately follow passage through the β-selection checkpoint. These results are strikingly similar to those from Egr-1 and Egr-3 transgenic mice, which also show increased numbers of DN4 and ISP cells (Miyazaki, 1997; Xi and Kersh, 2004), and are consistent with the notion that Egr proteins have overlapping roles during thymocyte development (Carter *et al.*, 2007). As observed for Egr-1 and Egr-3 (Miyazaki, 1997; Xi and Kersh, 2004), constitutive expression of Egr-2 appears to inhibit further development of these ISP cells. However, there are some DP cells and $CD4^+$ and $CD8^+$ SP cells suggesting that this block is incomplete. There are two distinct possible explanations for these observations both of

which have some support from the literature. The first is that constitutive Egr-2 expression in ISP cells directly impairs the further development of the ISP cells. Supporting this idea, Kersh and colleagues found that constitutive Egr-3 expression impaired both the expression and the activity of the transcription factor RORγt (Xi *et al.*, 2006) which is important for the DP cell phenotype (He *et al.*, 2000; Kurebayashi *et al.*, 2000; Sun *et al.*, 2000). As the authors of that study noted, this model implies that silencing of Egr protein expression in ISP cells is required for efficient progression to the DP stage (Xi *et al.*, 2006). The other possibility is that constitutive expression of Egr-2 permits the development of some cells that do not have a correctly rearranged TCR β chain and would normally fail the β-selection checkpoint. These cells develop to the ISP stage but are unable to develop further due to a lack of other, presumably pre-TCR derived, signals. In contrast, those cells that have a correctly rearranged TCR β chain and hence a pre-TCR complex may continue to the next stage of development as normal despite the constitutive expression of Egr-2. So rather than inhibiting the development of the ISP cells, this scenario envisages that constitutive Egr-2 expression drives the further development of cells that would otherwise fail β-selection and implies that these ISP cells would never progress to the DP stage as they are functionally deficient. Consistent with this possibility, constitutive expression of Egr-1 on a RAG-$1^{-/-}$ background permitted the development of cells to the DN4 and ISP stages but no further, suggesting that additional signals are required for maturation to the DP stage (Miyazaki, 1997). While circumstantial evidence (P Wang, personal communication) may tend to support the former possibility, the latter possibility cannot yet be formally excluded. Analysis of the TCR β loci in these ISP cells in Egr-2 cKO mice should help to definitely distinguish between these two hypotheses.

4.2 Egr-2 in peripheral T cells

Despite the apparently normal T cell development in Egr-2 cKO mice, we found that the total number of peripheral $CD4^+$ and $CD8^+$ T cells in three month old mice was reduced to around two-thirds of the level of age matched Egr-$2^{loxP/loxP}$ mice. While the expression of activation markers by peripheral $CD4^+$ T cells was largely normal in young Egr-2 cKO mice, there was a slight increase in the proportion of cells expressing high levels of the marker CD44. The roles of Egr-2 in $CD44^{high}$ cells are discussed further below (see section 4.4).

In the periphery of Egr-2 Tg mice the most striking change is the presence of a large number of cells that appear to be $CD8^+$ ISP thymocytes. This population may represent $CD8^+$ ISP cells that have escaped from the thymus into the periphery. In support of this concept, recent discoveries have indicated that immature thymocytes are indeed exported from the thymus (Lambolez *et al.*, 2006).

However, the continued presence of this $CD8^+CD3^-$ population in the periphery raises another question: how do these $CD8^+CD3^-$ cells survive? For naïve T cells, covert interactions with peptide-MHC ligands, together with exposure to cytokines such as IL-7, is required for their homeostasis and survival in the periphery (Kirberg *et al.*, 1997; Ernst *et al.*, 1999; Seddon and Zamoyska, 2002; Surh and Sprent, 2008). Since these $CD8^+CD3^-$ cells do not express a TCR complex there can be no TCR signalling so one would expect that these cells would slowly disappear over time, particularly in older mice which have decreased thymic output. However, we did not observe a diminution of this population in older Egr-2 Tg mice (P Wang, personal communication). While the aberrant nature of these cells means they may well have altered homeostatic

requirements, an intriguing possibility is that Egr-2 expression itself may help to facilitate the survival of these cells. Since Egr-2 is expressed by TCR signals it could be one of the downstream effectors of the survival signal provided by covert interactions with peptide-MHC ligands. Thus constitutive Egr-2 expression in these $CD8^+CD3^-$ cells may provide this signal which, in conjunction with homeostatic cytokines, permits the persistence of $CD8^+CD3^-$ cells in the periphery. Conversely in Egr-2 cKO mice, the lack of Egr-2 in naïve T cells may impair their survival resulting in the decreased numbers of peripheral T cells observed in young Egr-2 cKO mice. Consistent with this idea, Egr-1 has been reported to be induced in TCR Tg T cells upon interaction with APC in the absence of exogenous antigen, and Egr-1 KO mice also reportedly have decreased numbers of naïve splenic T cells (Bettini *et al.*, 2003). Further experiments will be needed to test this hypothesis. In particular, it would be interesting to transfer naïve Egr-2 Tg TCR Tg T cells into MHC deficient hosts to determine whether they have a relative survival advantage compared to naïve wild type TCR Tg T cells.

4.3 Egr-2 in T cell activation

Over-expression of Egr-2 in T cell lines results in reduced production of IL-2 after TCR stimulation and a slight up-regulation of the E3 Ubiquitin ligase Cbl-b (Safford *et al.*, 2005). Based on these findings, the authors of that study hypothesised that the mechanism for Egr-2 in maintaining T cell tolerance was down-regulation of TCR signalling. However, we did not detect a difference between naïve Egr-2 cKO and naïve $Egr\text{-}2^{loxP/loxP}$ T cells in terms of ERK MAPK activation following TCR stimulation *in vitro*. In addition, we did not observe hyper-proliferation of naïve Egr-2 cKO T cells in response to primary TCR stimulation *in vitro*, which would be expected if Egr-2 negatively regulated TCR signalling. On the contrary, we found that naïve Egr-2 cKO T

cells were consistently slightly hypo-responsive to primary TCR stimulation. This data suggests that, rather than simply inhibiting T cell activation as has been proposed (Safford *et al.*, 2005), Egr-2 has a more complex role in T cell activation.

One possible explanation for this result is that Egr-2 expression provides some protection from apoptosis rendering Egr-2 cKO cells more susceptible to AICD. Although we did not detect increased apoptosis after T cell activation, suggesting that the impaired proliferation of Egr-2 cKO cells is not due to increased cell death, we have not exhaustively examined all time points after stimulation and cannot formally exclude this possibility. Indeed, the microarray found that the expression of the anti-apoptotic gene Bcl-2 was slightly decreased in stimulated Egr-2 cKO cells compared to stimulated Egr-$2^{loxP/loxP}$ cells; which is consistent with this hypothesis. Furthermore, recently it has been reported that Egr-2 is required for maximal induction of Bcl-2 during positive selection (Lauritsen *et al.*, 2008). Future studies will be needed to definitely determine whether, and at what stages, Egr-2 cKO cells are more susceptible to AICD and the mechanisms involved.

4.4 Egr-2 in CD44^{high} cells

While we and others had previously found that Egr-2 is induced in tolerant T cells following TCR stimulation (Safford *et al.*, 2005; Anderson *et al.*, 2006 - see also Figure 3.1), the populations that express Egr-2 *in vivo* in normal mice were unknown. We have now demonstrated that Egr-2 is normally expressed in $CD44^{high}$ T cells *in vivo* in the absence of overt antigen stimulation. In addition, we found that Egr-2 deficiency results in the hyper-proliferation and progressive accumulation of $CD44^{high}$ T cells such that these cells became the predominant population in older Egr-2 cKO mice. Collectively,

this data suggests that Egr-2 is involved in controlling the homeostasis of CD44^{high} T cells *in vivo*.

4.4.1 Egr-2 in the homeostasis of CD44^{high} cells

CD44 is low on naïve cells and up-regulated after activation; thus, CD44^{high} cells are generally considered to be effector or memory cells (see Surh and Sprent, 2008). Although CD44^{high} cells are generated in response to antigen stimulation, CD44^{high} cells are also found in TCR Tg mice in the absence of cognate antigen stimulation and in mice fed low molecular weight diets (see Surh and Sprent, 2008). The CD44^{high} T cells in these mice are presumably generated in response to environmental antigens, such as commensal flora, or self-antigen (see Surh and Sprent, 2008). Therefore the responses of CD44^{high} T cells have to be controlled to prevent the possible development of autoimmune or hypersensitivity reactions. In contrast to the resting CD44^{low} naïve T cells, CD44^{high} cells undergo intermittent proliferation in response to homeostatic stimuli such as peptide-MHC and cytokines (Seddon *et al.*, 2003; Boyman *et al.*, 2006; Robertson *et al.*, 2006; Purton *et al.*, 2007; Surh and Sprent, 2008). The finding that Egr-2 is expressed by CD44^{high} cells in the absence of overt antigen stimulation suggests that covert homeostatic interactions with peptide-MHC, or perhaps cytokine signalling, may induce Egr-2. We hypothesise that the expression of Egr-2 in CD44^{high} cells may be induced from these homeostatic stimuli to control the proliferation of CD44^{high} T cells.

However, while the expression of Egr-2 was significantly increased in CD44^{high} cells compared to CD44^{low} cells, this finding does not necessarily mean that CD44^{low} cells do not express Egr-2 in response to homeostatic stimuli. This decreased expression of Egr-

2 in CD44^{low} cells may simply reflect the fact that CD44^{low} cells are apparently less responsive than CD44^{high} cells to antigen stimulation *in vivo* (Veiga-Fernandes *et al.*, 2000). Indeed, as outlined above (see section 4.2) some of the unexpected findings from Egr-2 cKO and Tg mice could perhaps be explained by a role for Egr-2 in the homeostasis of naïve T cells by supporting their survival.

Thus in Egr-2 cKO mice, naïve CD44^{low} T cells appear hypo-responsive while effector CD44^{high} T cells are hyper-responsive. At first it may seem peculiar that naïve Egr-2 cKO T cells are hypo-responsive while CD44^{high} T cells are hyper-responsive. However, it is not without precedent that a given transcription factor should have apparently different roles in these disparate cell types. Indeed, mice with a mutation in a critical enzyme in the NF-κB2 pathway had conflicting reports – with some groups reporting enhanced, and others diminished, responses to stimulation – which were resolved by the finding that the CD44^{low} cells are hyper-proliferative while the CD44^{high} cells are hypo-proliferative and enact a suppressor function on CD44^{low} cells in co-culture (Ishimaru *et al.*, 2006). This insight was only obtained by examining the responses of these two populations individually. Therefore, to definitively determine the effects of Egr-2 in CD44^{low} and CD44^{high} cell proliferation in response to TCR engagement, these cells should be isolated and stimulated separately. To date we have not been able to do this due to technical difficulties and this experiment remains for future investigation.

Based on the expression of phenotypic markers, such as CD44 and CD62L, it is not possible to definitively determine whether these hyper-reactive CD44^{high} cells are effector or memory cells. Therefore, whether Egr-2 primarily affects effector or memory cells, or affects both equally, remains to be established. The development of TCR transgenic Egr-2 cKO mice may help to resolve this question.

In our Tg4 model of tolerance the T cells were first activated with antigen *in vivo* and then rendered tolerant by persistent antigen stimulation (Anderson *et al.*, 2006). Similarly, the other models that found Egr-2 expression in tolerant or anergic cells either used T cells that were first activated *in vivo* by antigen (Safford *et al.*, 2005) or T cell lines that were maintained *in vitro* by intermittent antigen stimulation (Harris *et al.*, 2004). In all of these scenarios the cells would be more akin to $CD44^{high}$ effector cells rather than naïve $CD44^{low}$ cells. In light of these considerations, it is perhaps not surprising that we find that Egr-2 is expressed in $CD44^{high}$ cells *in vivo*. Therefore, collectively this data suggests that Egr-2 is involved in the maintenance of the self tolerance of $CD44^{high}$ cells *in vivo*.

4.5 Lupus-like autoimmunity in Egr-2 cKO mice

Consistent with the idea that Egr-2 is involved in self tolerance, we found that Egr-2 cKO mice developed a late onset lupus-like autoimmune disease. This disease was characterised by proteinuria, infiltration of T cells into internal organs, serum anti-nuclear antibodies and glomerulonephritis. Although we only observed clinical symptoms in older Egr-2 cKO mice we did find some signs of autoimmunity in younger mice suggesting that perhaps disease development is a gradual process. We observed a progressive increase in the serum titre of auto-antibodies directed against dsDNA and histone as the mice aged which was concomitant to the increase in the percentages of IFN-γ and IL-17 producing $CD44^{high}$ T cells.

FasL has been reported to be regulated by Egr-2 (Mittelstadt and Ashwell, 1999; Rengarajan *et al.*, 2000) and FasL and Fas mutant mice on certain genetic backgrounds

develop lupus-like autoimmune disease (Watanabe-Fukunaga *et al.*, 1992; Takahashi *et al.*, 1994; Nagata and Suda, 1995) suggesting the possibility that the autoimmunity in Egr-2 cKO mice is due to defective FasL expression. However, the diseases in FasL and Fas mutant mice on these backgrounds are characterised by the presence of aberrant $CD3^+CD4^-CD8^-B220^+$ cells (see Nagata and Suda, 1995); we did not detect any cells with a $CD3^+CD4^-CD8^-B220^+$ phenotype in Egr-2 cKO mice at any stage. In addition, we did not detect altered FasL expression in Egr-2 cKO cells; perhaps due to compensation by Egr-3 (Mittelstadt and Ashwell, 1998; Rengarajan *et al.*, 2000). These findings suggest that there are distinct mechanisms for lupus-like disease in Egr-2 cKO and FasL mutant mice.

It has been reported that Cre can recognise and catalyse recombination between pseudo-loxP sites, endogenous DNA sequences present in mammalian genomes that resemble loxP sites, *in vitro* (Thyagarajan *et al.*, 2000). Furthermore, one report using transgenic mice expressing Cre have found that Cre expression alone, i.e. in the absence of exogenous loxP sites, can have deleterious consequences during spermatogenesis *in vivo* (Schmidt *et al.*, 2000). Nevertheless, $CD4^+$ T cells from hCD2 Cre^+ mice had normal responses to TCR stimulation and no autoimmunity was observed in hCD2 Cre^+ mice as old as 20 months (P Wang, personal communication) suggesting that the phenotypes seen in Egr-2 cKO mice are due to Egr-2 deletion. Consistent with this, defective Egr-2 and Egr-3 expression has been observed in both spontaneous and experimentally induced lupus models (Sela *et al.*, 2008).

4.6 Homeostasis and tolerance

Notably, several models that have perturbed T cell homeostasis also develop late onset autoimmunity (e.g. Balomenos *et al.*, 2000; Murga *et al.*, 2001; Salvador *et al.*, 2002; Fanzo *et al.*, 2006; Arias *et al.*, 2007) as we observed in Egr-2 cKO mice. This raises the possibility that the observed perturbation of homeostasis may be a precursor to the development of autoimmunity. Although traditionally investigated using separate model systems, self-tolerance is important for homeostasis and *vice versa*.

Firstly, the peptide-MHC ligands that are important for the homeostasis of certain T cell subsets are ligands to which the T cells should be tolerant, which is established by central and/or peripheral tolerance mechanisms. If the T cells were not tolerant to these antigens, then not only might it lead to autoimmunity or hypersensitivity but it would also perturb T cell homeostasis. I should point out that for the purposes of this discussion the nature of the antigen, whether it is derived from self-antigen or environmental antigen, is irrelevant as both autoimmunity and hypersensitivity are perturbations of homeostasis. Hence the tolerance of T cells to such antigens is also important for their homeostasis in the periphery. Secondly, the process of adaptation itself is effectively a homeostatic mechanism, albeit a pre-emptive one. This is best demonstrated by contrasting the response of tolerant cells with the initial activation of T cells by antigen. Primary T cell activation results in an expansion of that T cell clone and hence an increase in total T cell numbers. This clonal expansion is then followed by a contraction of that clone, once the inciting antigen has been cleared, which acts to return total T cell numbers to normal – a well characterised homeostatic mechanism. In contrast, persistent stimulation in adaptive tolerance results in a progressive down-regulation of the response of the T cells such that the T cells eventually show only

minimal proliferation in response to antigen (Tanchot *et al.*, 2001; Singh and Schwartz, 2003; Anderson *et al.*, 2006). Hence the process of adaptation turns an agonist signal that initially induces strong proliferation into one that does not. Viewed in these terms the process of adaptation is a homeostatic mechanism as it serves to oppose an increase in total T cell numbers. Thirdly, both homeostasis, to some extent depending on the cell type, and tolerance are dependent upon the persistence of peptide-MHC. Many T cells, notably naïve T cells and some subsets of effector cells, are partly dependent on peptide-MHC derived signals for their homeostasis in the periphery (Kirberg *et al.*, 1997; Ernst *et al.*, 1999; Seddon and Zamoyska, 2002; Seddon *et al.*, 2003; Boyman *et al.*, 2006; Robertson *et al.*, 2006; Surh and Sprent, 2008). Equally, the persistence of antigen is required for the maintenance of adaptive tolerance in the periphery (Tanchot *et al.*, 2001; Singh and Schwartz, 2003). Therefore, at least for some cells, the maintenance of both homeostasis and tolerance depend on peptide-MHC derived signals. Furthermore, the biochemical signals that are presumably induced by peptide-MHC in these two instances are similar. The biochemical alterations in tolerant cells (Anderson *et al.*, 2006; Chiodetti *et al.*, 2006) resemble somewhat the pattern of phosophorylation observed in freshly isolated T cells *ex vivo* (van Oers *et al.*, 1993; Seddon and Zamoyska, 2002); the latter generally considered to be induced as a result of homeostatic stimuli. In both cases there is sub-maximal phosphorylation of TCR proximal signalling molecules but no detectable activation of signalling pathways such as Ca^{2+}/NFAT (van Oers *et al.*, 1993; Asai *et al.*, 2002; Seddon and Zamoyska, 2002; Anderson *et al.*, 2006; Chiodetti *et al.*, 2006). These considerations suggest that perhaps those same signals that are involved in the maintenance of certain T cell populations in the periphery are also responsible for the maintenance of the tolerance of the T cells towards those very same ligands. Therefore, if part of that signal were lacking it might

affect both homeostasis and tolerance and perhaps this is why many models with perturbed homeostasis also develop autoimmunity.

In lymphopaenia there is a relative increase in the level of available cytokines, access to APC and peptide-MHC due to the absence of competing cells and this induces vigorous "homeostatic" proliferation presumably due to increased stimulation. However, tolerant T cells do still proliferate if faced with a greatly increased level of antigen (Tanchot *et al.*, 2001; Schwartz, 2003; Singh and Schwartz, 2003) suggesting that the increased stimulation that the cell receives in lymphopaenia may also lead to a loss of tolerance to that antigen and an autoimmune reaction. This implies that the inertia of the T cell populations in normal conditions is an important factor in the maintenance of T cell tolerance. Supporting this idea, autoimmunity has been reported to be accompanied by lymphopaenia in both mouse models and patients (Zandman-Goddard and Shoenfeld, 2002; King *et al.*, 2004). As the size of the total T cell population is controlled by homeostasis, then T cell homeostasis is a factor in the maintenance of T cell tolerance. So therefore, homeostasis and tolerance work together to maintain total T cell numbers and to control potentially autoreactive populations. Our data suggest that Egr-2 is an intrinsic effector involved in both homeostasis and tolerance and support the idea that homeostasis and tolerance are not discrete phenomena but are two overlapping facets of T cell biology.

4.7 Other Egr-2 KO data

Recently another group reported their findings from Egr-2 deficient T cells which initially appear to conflict somewhat with our results from Egr-2 cKO mice. They used foetal liver cells from TCR Tg Egr-2 KO mice to reconstitute the immune system of

irradiated Balb/c mice and found that these Egr-2 KO T cells were hyper-responsive to TCR stimulation (Collins *et al.*, 2008). However, the lymphopaenia induced by irradiation would provoke vigorous "homeostatic" proliferation of the transferred TCR Tg Egr-2 KO T cells and conversion to $CD44^{high}$ effector phenotype (see Surh and Sprent, 2008). Therefore, these cells are likely to be more akin to the $CD44^{high}$ T cells that are hyper-proliferative in Egr-2 cKO mice *in vivo* rather than the primary quiescent naïve T cells we examined *in vitro*. When compared to the $CD44^{high}$ *in vivo* proliferation results, the results from this paper are consistent with the findings from Egr-2 cKO mice. Similarly, the previous study from this group examining the effects of Egr-2 over-expression (Safford *et al.*, 2005) mentioned above (see section 4.3) used a methodology that required that the T cells were pre-activated *in vitro*, rather than quiescent naïve T cells, and hence all the results from this group are consistent with the findings from Egr-2 cKO mice.

The more recent paper by these authors proposed that part of the mechanism of Egr-2 and Egr-3 function was to inhibit Egr-1 expression and thereby inhibit T cell activation (Collins *et al.*, 2008). However, this hypothesis is dependent on Egr-1 having a positive role in T cell activation; this is not supported by T cells from Egr-1 KO mice, which show normal responses to TCR stimulation (Lee *et al.*, 1995; Singh *et al.*, 2004). We found no evidence for cross-regulation of Egr proteins so far in our model, although we did not extensively investigate this. In any case, the importance of any potential cross-regulation between the Egr proteins remains to be determined.

Even more recently, another paper used a different method to generate Egr-2 deficient T cells (Lauritsen *et al.*, 2008). They also used $Egr\text{-}2^{loxP/loxP}$ mice but instead of using the hCD2 construct to drive Cre expression they used the Lck promoter. This promoter directs transgene expression at an earlier stage than the hCD2 construct, with Lck driven

transgene expression detectable even in the earliest stages of T cell development such as DN1 cells (reviewed in Cantrell, 2002). Using these mice they found that Egr-2 was necessary for efficient positive selection but not for negative selection (Lauritsen *et al.*, 2008). Notably, exactly the same results were previously seen in Egr-1 KO mice (Bettini *et al.*, 2002); suggesting that this is another area where there is overlap between Egr proteins in terms of function. As stated above we did not see this phenotype in our Egr-2 cKO mice; perhaps due to incomplete deletion in our model at this stage (see section 4.1 above). The authors observed a small decrease in Bcl-2 expression in their conditional Egr-2 KO model (Lauritsen *et al.*, 2008). Consistent with this we also found slightly decreased expression of Bcl-2 by Egr-2 cKO T cells and, as stated above, some of the unexpected results from Egr-2 cKO and Tg mice could perhaps be explained by a pro-survival effect of Egr-2. So overall, the results from this model are consistent with our findings from our Egr-2 cKO mice.

4.8 Mechanisms of Egr-2 function

4.8.1 Egr-2 regulation of p21cip1

We found that Egr-2 regulates the expression of p21cip1; a negative regulator of cell cycle progression (see Sherr and Roberts, 1999). Notably defective expression of p21cip1 has been implicated in lupus-like disease (Balomenos *et al.*, 2000; Salvador *et al.*, 2002; Arias *et al.*, 2007) although this is controversial (Santiago-Raber *et al.*, 2001; Lawson *et al.*, 2002; Lawson *et al.*, 2004). A study of p21cip1 KO mice on a mixed 129/Sv × C57BL/6 background found that these mice developed a lupus-like autoimmune disease (Balomenos *et al.*, 2000). However, another group reported that p21cip1 KO mice on a different mixed 129/Sv × C57BL/6 background developed only a

mild lupus-like autoimmune disease (Santiago-Raber *et al.*, 2001). This group subsequently found that p21 deficiency in mice of the lupus prone BXSB background reduced the incidence of disease in male mice (Lawson *et al.*, 2004). Based on *in vitro* findings of increased FasL mediated apoptosis, the authors attributed this resistance to disease to increased apoptosis of p21cip1 deficient T cells. However, the incidence of disease in female BXSB mice was not affected by p21cip1 deficiency but T cells from these mice showed similar increases in apoptosis (Lawson *et al.*, 2002). The BXSB model of lupus is unusual as male mice are more significantly affected which is attributed to a region on the Y chromosome termed the Yaa (Y chromosome Accelerator of Autoimmunity) (Pisitkun *et al.*, 2006). Taken together, this data from BXSB mice suggests that it is the combination of the Yaa with p21 deficiency that impairs lupus development in male p21cip1 KO BXSB mice, rather than increased apoptosis of p21cip1 deficient T cells (Arias *et al.*, 2007). While progress has recently been made in identifying the genetic abnormalities responsible for the disease promoting effects of Yaa (Pisitkun *et al.*, 2006; Deane *et al.*, 2007), how the gene products interact with p21cip1, and which cell types are involved, is a matter of speculation. Indeed, whether p21cip1 deficiency does result in increased apoptosis has been called into question; a recent study of p21cip1 KO mice on the C57BL/6 background found that apoptosis induction was not affected (Arias *et al.*, 2007). Thus, the increased apoptosis seen in p21cip1 KO BXSB mice may be specific to the BXSB background rather than a general effect of p21cip1 deficiency. Furthermore, p21cip1 KO mice on the C57BL/6 background also develop lupus-like autoimmune disease (Arias *et al.*, 2007), although this was not as severe as that seen in the mixed background mice (Balomenos *et al.*, 2000). Therefore, collectively these data suggest that defective p21cip1 expression may be part of the mechanism responsible for the breakdown of self-tolerance in Egr-2 cKO mice. In particular, the defect in expression

of p21cip1 provides a potential explanation of the hyper-proliferation and accumulation of $CD44^{high}$ T cells *in vivo* in Egr-2 cKO mice, since this also was observed in p21cip1 deficient mice on the C57BL/6 background (Arias *et al.*, 2007).

However, whether defective expression of p21cip1 can fully explain the loss of tolerance in Egr-2 cKO mice is unclear. In particular, it is difficult to envisage how defective p21cip1 expression leads to the inflammation observed in Egr-2 cKO mice. Since Egr-2 is a transcription factor it could potentially regulate hundreds of genes, any number of which might be involved in the maintenance of tolerance. Thus, there are likely to be other genes regulated by Egr-2 that are relevant to autoimmune disease development. We performed a microarray to try and identify novel targets of Egr-2.

4.8.2 Microarray analysis of Egr-2 cKO T cells

Of those genes that were differentially expressed according to the microarray the most common functional category was metabolic enzymes; more than a quarter of the genes with known function fell into this category. Interestingly, a similar effect on metabolic genes was previously reported in Egr-1, Egr-3 DKO mice (Carter *et al.*, 2007).

The microarray also revealed that the expression of several key effector cytokines was altered in stimulated Egr-2 cKO T cells compared to their $Egr\text{-}2^{loxP/loxP}$ counterparts with IL-4 and IL-13 decreased and IFN-γ and, in particular, IL-17A and IL-17F increased (discussed further below).

In addition to the increased expression of IL-17A and IL-17F the microarray also showed increased expression of other molecules that have been reported to be expressed

by, and/or to be involved in the differentiation of, IL-17 producing $CD4^+$ T cells. Among these, the chemokine receptor CCR6 is involved in trafficking to inflamed sites and has been found to be expressed by IL-17 producing $CD4^+$ T cells in a mouse model of Rheumatoid Arthritis (Hirota *et al.*, 2007). In addition, the pro-inflammatory cytokine IL-1α has been reported to promote the development of IL-17 producing $CD4^+$ T cells, at least *in vitro* (Sutton *et al.*, 2006). While IL-1α was originally described as a cytokine produced by macrophages, IL-1α expression by some T cell subsets is now well documented (van Kooten *et al.*, 1991; van Rietschoten *et al.*, 2006). Finally, autocrine production of IL-21 has been implicated in the proliferation and/or lineage commitment of IL-17 producing $CD4^+$ T cells (Korn *et al.*, 2007; Nurieva *et al.*, 2007; Zhou *et al.*, 2007).

We also found increased expression of the anti-inflammatory cytokine IL-10 by Egr-2 cKO T cells. Although it is anti-inflammatory, IL-10 has been implicated as having a pathogenic role in lupus (see Moore *et al.*, 2001) and so this increased IL-10 expression could be relevant to the autoimmunity observed in Egr-2 cKO mice.

IL-15 is an IL-2 related cytokine that is important for T cell homeostasis (see Surh and Sprent, 2008). It was somewhat surprising to find increased expression of this cytokine on the microarray, however, since traditionally T cells were not considered to be a source of IL-15 *in vivo* (see Fehniger and Caligiuri, 2001). Nevertheless, there is one report that has demonstrated that IL-15 is expressed by T cells (Azimi *et al.*, 1998; Fehniger and Caligiuri, 2001). However, there are multiple post-transcriptional mechanisms that serve to regulate IL-15 expression (see Fehniger and Caligiuri, 2001) so the increased expression of this cytokine needs to be confirmed at the protein level. Furthermore, the biological relevance of the increased expression of this cytokine

remains to be determined as the importance of T cell production of IL-15 *in vivo* is unclear. These arguments notwithstanding, one could envisage that increased expression of IL-15 could also contribute to the hyper-proliferation of $CD44^{high}$ T cells in Egr-2 cKO mice *in vivo* by promoting homeostatic proliferation and/or supporting cell survival (Fehniger and Caligiuri, 2001; Boyman *et al.*, 2006; Purton *et al.*, 2007; Surh and Sprent, 2008). In addition to its well characterised role in T cell homeostasis, IL-15 is a potent T cell chemoattractant and increased IL-15 levels have been observed in several models of, and patients with, inflammatory autoimmune processes (see Fehniger and Caligiuri, 2001). While the precise roles played by IL-15 in inflammation remain unclear, increased IL-15 expression could also be involved in the development of autoimmunity in Egr-2 cKO mice.

4.8.3 Effector T cell subsets in Egr-2 cKO mice

Consistent with the 3 fold reduction in T_H2 cytokines detected by microarray and Real Time RT-PCR, the numbers of IL-4 producing $CD4^+$ T cells were decreased in Egr-2 cKO mice. Conversely, the numbers of IFN-γ producing $CD4^+$ T cells were increased in Egr-2 cKO mice consistent with the 2 fold increase in IFN-γ mRNA expression. Similarly, IL-17 producing $CD4^+$ T cells were also increased, which was by far the most marked alteration in terms of expression of effector cytokines by $CD4^+$ T cells in Egr-2 cKO mice, consistent with the 3-4 fold increase in IL-17A and IL-17F mRNA levels.

Notably, the increased numbers of IFN-γ and IL-17 producing $CD4^+$ T cells in Egr-2 cKO mice further increased as the Egr-2 cKO mice aged. In addition, we found that the IFN-γ dependent IgG2a isotype was increased in the serum of Egr-2 cKO mice. These findings suggest that IFN-γ and IL-17 producing $CD4^+$ T cells may be involved in the

development of the autoimmune disease observed in Egr-2 cKO mice. Consistent with this possibility, IFN-γ is important for the development of lupus-like disease (Balomenos *et al.*, 1998) while IL-17 producing $CD4^+$ T cells have recently been implicated in auto-antibody production (Hsu *et al.*, 2008; Wu *et al.*, 2008).

4.9 Future Work

The work presented here raises several interesting avenues for future investigation. For Egr-2 cKO mice the mechanism by which Egr-2 deficiency leads to increased production of inflammatory cytokines is the key issue. Most of the evidence indicates that it is the $CD44^{high}$ cells that are producing these cytokines but this has not been validated for cytokine mRNA. Isolation of $CD44^{low}$ and $CD44^{high}$ cells and separate stimulation should definitively resolve this. *In vitro* $T_H1/T_H2/T_H17$ polarisation and further investigation of TCR and cytokine signalling pathways may facilitate future investigation of the molecular mechanism of Egr-2 in control of inflammatory cytokine production and/or effector T cell differentiation. Isolation of $CD44^{low}$ and $CD44^{high}$ cells and separate stimulation should also help to resolve the issue of the role of Egr-2 in T cell activation in $CD44^{low}$ and $CD44^{high}$ cells and facilitate future investigation of the molecular mechanism of Egr-2 in T cell activation.

For Egr-2 Tg mice the mechanism by which constitutive Egr-2 overexpression leads to the observed thymocyte developmental defects is the key issue. As stated above analysis of the TCR β loci in ISP cells should assist with this and facilitate future investigation of the molecular mechanism of Egr-2 in T cell development.

4.10 Conclusions and model for Egr-2 function

Our work has confirmed a role for Egr-2 in T cell tolerance and revealed hitherto unknown roles for Egr-2 in T cell homeostasis and inflammatory responses…how can these disparate strands be weaved into a coherent picture of the role of Egr-2 in T cells?

Several studies in thymocytes suggest that Egr proteins may have a pro-survival effect both during β-selection (Carter *et al.*, 2007) and positive selection (Bettini *et al.*, 2002; Lauritsen *et al.*, 2008). In addition, there is one report that suggests that Egr-1 may also have a pro-survival role in peripheral naïve T cells (Bettini *et al.*, 2003). We found no direct evidence for a pro-survival role of Egr-2 in our study. However, some of the unexpected results from Egr-2 cKO and Tg mice; such as the mild hypo-proliferation of naïve Egr-2 cKO T cells *in vitro*, the small decrease in peripheral T cell numbers in young Egr-2 cKO mice and the persistence of aberrant cells that resemble ISP thymocytes in the periphery of Egr-2 Tg mice; could perhaps be explained by the possibility that Egr-2 may have a pro-survival effect in mature peripheral naïve T cells as outlined above (see sections 4.2 and 4.3). However, since there is no direct evidence from our study to support this hypothesis, I shall not include it in the model of Egr-2 function and instead focus on the role of Egr-2 in effector T cells.

Based on our findings presented here I have formulated a general model for Egr-2 function in effector T cells. This is that Egr-2 expression induces 2 effects; 1) retarding cell cycle progression and 2) restraining inflammatory responses by modulating differentiation (see Figure 4.1).

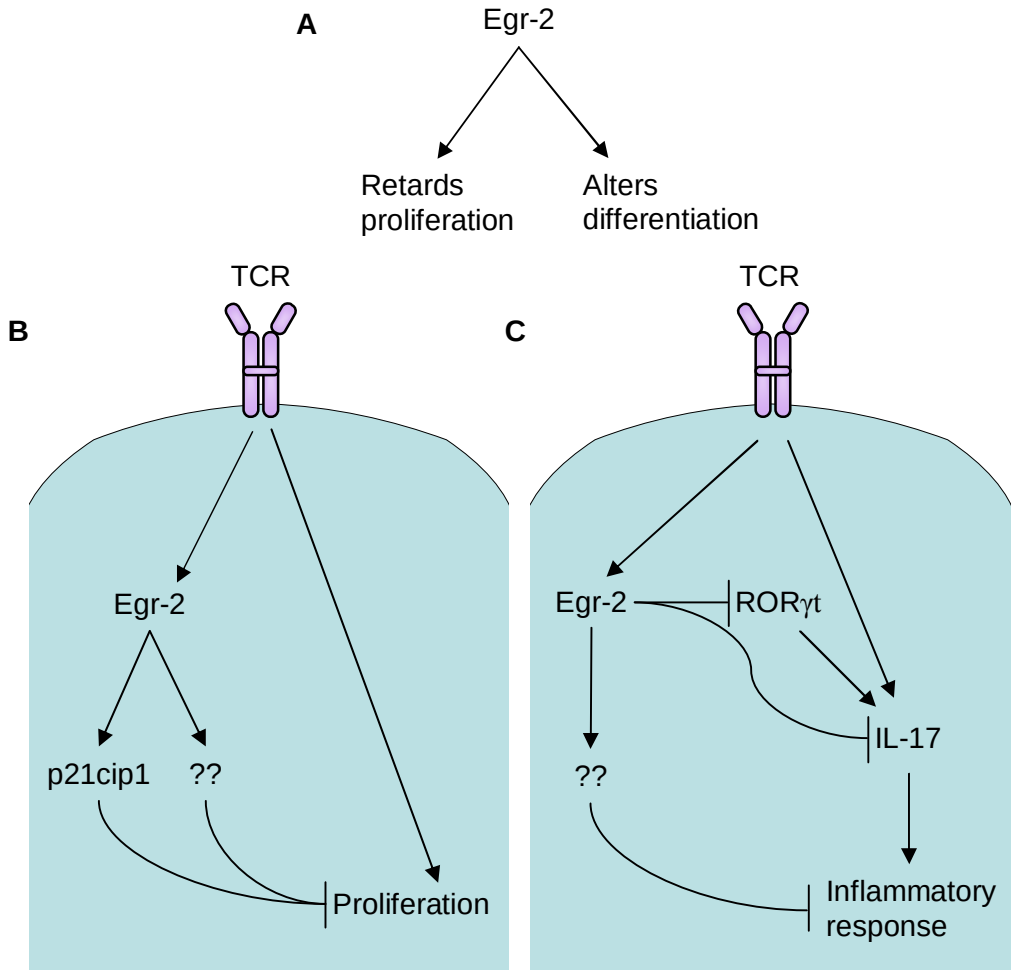

Figure 4.1: A model for Egr-2 function. (**A**) Proposed two effects of Egr-2. (**B**) Potential role of Egr-2 in retarding proliferation. We have shown that Egr-2 regulates p21cip1 expression (Figures 3.18 and 3.19). Via induction of negative regulators such as this Egr-2 could serve to retard proliferation. (**C**) Potential role of Egr-2 in modulating inflammatory responses. Egr-2 could modulate the expression or function of transcription factors, such as RORγt, that are important for the generation of IL-17 producing $CD4^+$ T cells, or directly interfere with IL-17 expression or function by some other as yet undiscovered mechanism to control the development of IL-17 producing $CD4^+$ T cells. Egr-2 could also have similar roles in the control of IFN-γ producing $CD4^+$ T cells (Not shown). By regulating the development of pro-inflammatory cytokine producing $CD4^+$ T cells Egr-2 could thereby control inflammatory responses. See text for further details.

The possibility that Egr-2 may supply an inhibitory signal downstream of the TCR which would serve as a brake on proliferation would explain the hyper-proliferation of

the $CD44^{high}$ cells in Egr-2 cKO mice *in vivo*. Presumably, this *in vivo* proliferation is induced by homeostatic stimuli and these stimuli also appear to induce Egr-2 in normal mice (see Figure 3.12). Our model predicts that in normal mice Egr-2 serves to limit proliferation but in the absence of Egr-2, in Egr-2 cKO mice, proliferation is unrestrained. This possibility would also explain the results of Powell and colleagues who found that Egr-2 over-expression inhibits the responses of T cell lines *in vitro* (Safford *et al.*, 2005) and the subsequent results from the same group that observed hyper-responsive Egr-2 KO T cells (Collins *et al.*, 2008). However, as argued above, given the systems used in the latter two studies the cells would presumably be more akin to $CD44^{high}$ cells rather than naïve $CD44^{low}$ cells. Therefore, all the evidence that supports this "proliferative brake" role for Egr-2 is from effector cells.

Controlling homeostatic proliferation of effector $CD44^{high}$ T cells is critical for T cell homeostasis. Therefore, our model predicts that Egr-2 is important for T cell homeostasis. The progressive accumulation of $CD44^{high}$ T cells *in vivo* as the mice age supports this idea.

In all other cell types examined Egr-2 has been associated with differentiation (Voiculescu *et al.*, 2001; Parkinson *et al.*, 2004; Laslo *et al.*, 2006). In T cells, Egr-2 has likewise been implicated in thymocyte differentiation; originally in β-selection and more recently in positive selection (Carleton *et al.*, 2002; Carter *et al.*, 2007; Lauritsen *et al.*, 2008). In mature T cells however, there is no published evidence of Egr-2 involvement in effector T cell differentiation. Our discovery of increased numbers of IFN-γ and IL-17 producing $CD4^+$ T cells in Egr-2 cKO mice suggest that Egr-2 may be involved in effector T cell differentiation. Notably, Egr-1 and Egr-2 have been reported to be involved in myeloid cell fate specification by promoting the macrophage

phenotype and inhibiting the competing neutrophil gene expression programme (Laslo *et al.*, 2006) and a similar role has been proposed for Egr-2 in rhombomere differentiation (Voiculescu *et al.*, 2001); cell lineage decisions that are, in many ways, analogous to effector T cell differentiation.

Given that we observed increased IFN-γ and IL-17 mRNA expression by activated T cells from Egr-2 cKO mice one could speculate that Egr-2 mediates some of its effects by regulating cytokine expression. However, the T cells used in this experiment were total T cells not purified naïve T cells and would therefore include some effector cells. We observed increased numbers of IFN-γ and IL-17 producing $CD4^+$ T cells in Egr-2 cKO mice of comparable ages to those used in the above experiment suggesting that the increased IFN-γ and IL-17 mRNA expression may be due to the presence of increased numbers of IFN-γ and IL-17 producing $CD4^+$ T cells rather than indicating that Egr-2 affects cytokine expression *per se*. Nevertheless, there is some support for the idea that Egr-2 may regulate cytokine expression from the work of Skerka and colleagues who reported that Egr proteins were directly involved in modulating cytokine expression via interaction with NF-κB and NFAT (Decker *et al.*, 1998; Decker *et al.*, 2003; Wieland *et al.*, 2005). Also at least some of the key effector cytokines can influence the expression of cytokines by other $CD4^+$ T cells so if there was a primary effect on cytokine expression this could feedback and result in increased numbers of cytokine producing effector T cells. Therefore a role for Egr-2 in modulating IL-17, IL-4 or IFN-γ expression cannot be ruled out from the data at hand. However, we favour the idea that Egr-2 may be involved in effector cell differentiation as this is more in keeping with the roles of Egr-2 in other cell types (Voiculescu *et al.*, 2001; Parkinson *et al.*, 2004; Laslo *et al.*, 2006).

For T_H1 cells the transcription factor T-bet is important for the establishment of the IFN-γ secreting phenotype (Szabo *et al.*, 2000). Recently, the transcription factors RORα and RORγt have been identified as important for the development of IL-17 producing $CD4^+$ T cells (Ivanov *et al.*, 2006; Yang *et al.*, 2008). In addition, transcription factors that are activated downstream of cytokine signalling are also important for the development of cytokine producing $CD4^+$ T cells. Signal Transducer and Activator of Transcription (STAT)-1 and STAT-4 are important for the development of IFN-γ producing $CD4^+$ T cells, while STAT-3 is important for IL-17 producing $CD4^+$ T cells (see Dong, 2008). One could imagine that Egr-2 may modulate the expression and/or activity of one or more of these transcription factors. Intriguingly, Egr-3 has been reported to indirectly inhibit the expression of RORγt and to physically interact with RORγt protein inhibiting its activity (Xi *et al.*, 2006). Preliminary studies of T-bet and RORγt did not find any evidence for Egr-2 modulation of the expression or activity of these key transcription factors (P Wang, personal communication) although this cannot be formally ruled out. Another possibility is that Egr-2 may impact on other, as yet undiscovered, pathways that are involved in effector T cell differentiation. The exact nature of the mechanisms by which Egr-2 functions in effector T cell differentiation will require additional investigation.

In any case, the increased numbers of $CD4^+$ T cells producing pro-inflammatory cytokines observed in Egr-2 cKO mice indicates that Egr-2 is involved in the control of inflammatory responses. This role of Egr-2 may be important not only for self-tolerance but also to limit immunopathology during a productive immune response caused by over-zealous responses to foreign antigen. This may be particularly applicable to certain diseases, such as some viral lung diseases, where immunopathology rather than direct pathogen effects appear to be responsible for much of the pathology (Hussell *et al.*,

2001). This role of Egr-2 would also serve to maintain homeostasis, this time of the whole organism rather than specifically of T cells.

In adaptive tolerance T cell proliferation and effector functions are impaired (Tanchot *et al.*, 2001; Singh and Schwartz, 2003; Safford *et al.*, 2005; Anderson *et al.*, 2006). As Egr-2 expression has been observed in models of adaptive tolerance, by our group as well as others (Safford *et al.*, 2005; Anderson *et al.*, 2006), the above conjecture on the twin roles of Egr-2 in restraining proliferation and inhibiting inflammatory responses could potentially explain these two aspects of the tolerant T cell phenotype. Evidence for a role of Egr-2 in T cell tolerance is most obviously supplied by the autoimmunity in Egr-2 cKO mice.

Recently it has been reported that Egr-2, but not Egr-1 or Egr-3, is essential for the terminal maturation of NKT cells, a CD1 restricted subset of T cells that also express NK cell markers (Lazarevic *et al.*, 2009). Intriguingly, this group found that in the absence of Egr-2, NKT cell precursors displayed increased proliferation coupled to impaired differentiation (Lazarevic *et al.*, 2009). Importantly, this study demonstrates both phenotypic changes predicted by our model and therefore supports our hypothesis.

It is worth comparing our findings on the role of Egr-2 with the roles of NFAT and FoxP3; two transcription factors involved in T cell tolerance that have been extensively investigated. FoxP3 is constitutively expressed by T_{Reg} cells and is important for the development of the T_{Reg} phenotype (Fontenot *et al.*, 2003; Hori *et al.*, 2003). Thus FoxP3 is important for cell extrinsic mechanisms of T cell tolerance. In contrast, all the evidence, both from our study and others (Harris *et al.*, 2004; Safford *et al.*, 2005; Anderson *et al.*, 2006), supports the notion that Egr-2 is a transcription factor that is

involved in cell intrinsic mechanisms of T cell tolerance. While the transcription factor NFAT has also been reported to be important for cell intrinsic mechanisms of tolerance (Macian *et al.*, 2002; Heissmeyer *et al.*, 2004) there are differences in both the expression profile and proposed mechanism of action between NFAT and Egr-2. While both NFAT activation and Egr-2 expression is detectable in activated T cells, NFAT is not activated in adaptively tolerant T cells but Egr-2 is expressed (Anderson *et al.*, 2006 - see also Figure 3.1). Furthermore, perhaps the most striking difference between Egr-2 and NFAT is the proposed mechanism of action. NFAT has been reported to induce E3 ligase expression and thereby regulate TCR signalling and the T cell activation threshold (Heissmeyer *et al.*, 2004). While Egr-2 has been proposed to function in a similar way (Safford *et al.*, 2005) the evidence we found from Egr-2 cKO mice is inconsistent with this theory. Instead Egr-2 appears to regulate the homeostasis and inflammatory responses of effector T cells. However, the precise mechanisms by which Egr-2 functions are still not clear and will require further investigation. This role of Egr-2, while more subtle than the broad effects of NFAT and FoxP3, is arguably just as important for self-tolerance.

This general model for Egr-2 function could also apply to some of the other Egr proteins and could potentially explain some of the reported results; although there may well be some differences between individual Egr proteins in the extent of their involvement in particular roles. For example, while an inhibitory effect on proliferation could explain the hyper-reactivity of Egr-3 KO T cells and the inhibition of T cell responses by Egr-3 over-expression (Safford *et al.*, 2005; Collins *et al.*, 2008), there is no reported evidence of an inhibitory effect of Egr-1 on proliferation (Lee *et al.*, 1995; Singh *et al.*, 2004). Given this potential overlap between the Egr proteins, the importance of their function may only be apparent upon the establishment of double,

triple, or even quadruple KO mice. The importance of these potential roles of Egr proteins and the extent of overlap between them for each particular role will require further investigation.

To summarise our model we propose that Egr-2 retards proliferation and differentially promotes differentiation. These two effects may well be common roles for Egr-2 across many different biological systems, such as neuronal cells and myeloid lineages (Voiculescu *et al.*, 2001; Parkinson *et al.*, 2004; Laslo *et al.*, 2006), and may represent a general cell biological function. In effector T cells, Egr-2 mediated differential promotion of differentiation inhibits the development of a pro-inflammatory phenotype and thus Egr-2 serves to restrain pro-inflammatory responses. These two effects: retarding proliferation and restraining inflammatory responses, presumably through altered differentiation, therefore makes Egr-2 an intrinsic regulator of effector T cells which acts to preserve tolerance to self and environmental antigens. By regulating effector T cell responses in this way, Egr-2 also serves to promote homeostasis, both of the organism in general and of T cells in particular, and therefore Egr-2 enforces a pro-tolerogenic and pro-homeostatic gene expression programme.

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